

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/35, 39/39, A61P 37/04		A2	(11) International Publication Number: WO 00/54803
			(43) International Publication Date: 21 September 2000 (21.09.00)
(21) International Application Number: PCT/US00/07213 (22) International Filing Date: 16 March 2000 (16.03.00) (30) Priority Data: 60/124,595 16 March 1999 (16.03.99) US 60/125,071 17 March 1999 (17.03.99) US (71) Applicant: PANACEA PHARMACEUTICALS, LLC [US/US]; 640 Sasco Hill Road, Fairfield, CT 06430 (US). (72) Inventors: SOSIN, Howard, B.; 640 Sasco Hill Road, Fairfield, CT 06430 (US). CAPLAN, Michael, J.; 1217 Racebrook Road, Woodbridge, CT 06525 (US). (74) Agent: MAH, Stanley, C.; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>	
(54) Title: IMMUNOSTIMULATORY NUCLEIC ACIDS AND ANTIGENS			
(57) Abstract			
<p>The present invention provides methods and compositions for modulating an individual's immune response to antigens. It is an aspect of the present invention that allergic responses to antigens, which in some cases lead to asthma and even anaphylaxis, can be treated or prevented by administering compositions having immunostimulatory oligonucleotides having unmethylated CpG sequences. It is another aspect of the present invention that allergies to antigens, especially one that result in asthma and anaphylaxis, can be treated or prevented by administering compositions containing immunostimulatory oligonucleotides having unmethylated CpG dinucleotide sequences and further comprising antigen(s), fragments of the antigen, mixtures of fragments of the antigen, antigens modified to reduce Th2-type immune responses, and fragments of the antigen modified to reduce Th2-type immune responses. Cellular systems for studying immunostimulation by CpG containing nucleic acids include <i>in vivo</i>, <i>in vitro</i> or <i>ex vivo</i> systems.</p>			
BEST AVAILABLE COPY			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

IMMUNOSTIMULATORY NUCLEIC ACIDS AND ANTIGENS

This application claims priority under 35 U.S.C. 119(e) to the provisional application U.S.S.N. 60/124.595 entitled "Immunostimulatory Nucleic Acid Sequences Associated with Antigens" filed March 16, 1999 and to the provisional application U.S.S.N. 60/125.071 entitled "Nucleic Acid Delivery" filed March 17, 1999, both of which are incorporated in their entirety by reference.

Related applications

The present invention is generally in the area of immunomodulatory methods and compositions and in particular relates to methods and compositions for treating or preventing allergies. This application is related to U.S.S.N. 60/169.330 entitled "Controlled Delivery of Antigens" filed Dec. 6, 1999; U.S.S.N. 09/141,220 entitled "Methods and Reagents for Decreasing Clinical Reaction to Allergy" filed Aug. 27, 1998; U.S.S.N. 09/455,294 entitled "Peptide Antigens" filed December 6, 1999; US Patent Application filed January 28, 2000 entitled "Methods and Reagents for Decreasing Clinical Reaction to Allergy" by Bannon et al.; U.S.S.N. 60/124.595 entitled "Immunostimulatory Nucleic Acid Sequences Associated with Antigens" filed March 16, 1999; and U.S.S.N. 60/125.071 entitled "Nucleic Acid Delivery" filed March 17, 1999 the teachings of which are all incorporated herein by reference in their entirety.

Background of the Invention

Allergies and Asthma

Allergic reactions pose serious public health problems worldwide. Pollen allergy alone (allergic rhinitis or hay fever) affects about 10-15% of the population, and generates huge economic costs. For example, reports estimate that pollen allergy generated \$1.8 billion of direct and indirect expenses in the United States in 1990 (*Fact Sheet*, National Institute of Allergy and Infectious Diseases; McMenamin, *Annals of Allergy* 73:35, 1994). Asthma, which can be triggered by exposure to antigens, is also a serious public health problem, and like anaphylactic allergic reactions, can lead to death in extreme cases. Asthma currently accounts for millions

of visits yearly to hospitals and is increasing in frequency. The only treatment currently available is for alleviation of symptoms, for example, to relieve constriction of airways. More serious than the economic costs associated with pollen and other inhaled allergens (e.g., molds, dust mites, animal dander) is the risk of anaphylactic reaction observed with allergens such as food allergens, venoms, drugs, and rubber-based products.

Allergic reactions result when an individual's immune system overreacts, or reacts inappropriately, to an encountered antigen. Typically, there is no allergic reaction the first time an individual is exposed to a particular antigen. However, it is the initial response to an antigen that primes the system for subsequent allergic reactions. In particular, the antigen is taken up by antigen presenting cells (APC; e.g., macrophages and dendritic cells) that degrade the antigen and then display antigen fragments to T cells. T cells, in particular CD4⁺ "helper" T-cells, respond by secreting a collection of cytokines that have effects on other immune system cells. The profile of cytokines secreted by responding CD4⁺ T cells determines whether subsequent exposures to the antigen will induce allergic reactions. Two classes of CD4⁺ T cells (Th1 and Th2: T-lymphocyte helper type) influence the type of immune response that is mounted against an antigen.

The Th1-type immune response involves the stimulation of cellular immunity to antigens and infectious agents and is characterized by the secretion of IL-2, IL-6, IL-12, IFN γ , and TNF β by CD4⁺ T helper cells and the production of IgG antibodies. Exposure of CD4⁺ T cells to antigens can also activate the cells to develop into Th2 cells, which secrete IL-4, IL-5, IL-10 and IL-13. One effect of IL-4 production is to stimulate the maturation of B cells that produce IgE antibodies specific for the antigen. These antigen-specific IgE antibodies attach to receptors on the surface of mast cells, basophils and eosinophils, where they act as a trigger to initiate a rapid immune response to the next exposure to antigen. When the individual encounters the antigen a second time, the antigen is quickly bound by these surface-associated IgE molecules. Each antigen typically has more than one IgE binding site, so that the surface-bound IgE molecules quickly become crosslinked to one another through their simultaneous (direct or indirect) associations with antigen. Such cross-linking induces mast cell degranulation, resulting in the release of histamines and other

substances that trigger allergic reactions. Individuals with high levels of IgE antibodies are known to be particularly prone to allergies.

The Th1 and Th2-type responses are antagonistic. In other words, one response inhibits secretions characterized by the other immune response. Thus, therapies to control the Th1 and Th2-mediated immune responses are highly desirable to control immune responses to allergenic agents and to infectious agents. Current treatments for allergies involve attempts to "vaccinate" a sensitive individual against a particular allergen by periodically injecting or treating the individual with a crude suspension of the raw allergen. The goal, through controlled administration of known amounts of antigen, is to modulate the IgE response mounted in the individual. If the therapy is successful, the individual's IgE response is diminished, or can even disappear. However, the therapy requires several rounds of vaccination, over an extended time period (3-5 years), and very often does not produce the desired results. Moreover, certain individuals suffer anaphylactic reactions to the vaccines, despite their intentional, controlled administration.

Summary of the Invention

The present invention provides methods and compositions for modulating an individual's immune response to antigens. It is an aspect of the present invention that allergic responses to antigens, which in some cases lead to asthma and even anaphylaxis, can be treated or prevented by administering compositions having immunostimulatory oligonucleotides including unmethylated CpG sequences. It is another aspect of the present invention that allergies to antigens, especially ones that result in asthma and/or anaphylaxis, can be treated or prevented by administering compositions containing immunostimulatory oligonucleotides having unmethylated CpG dinucleotide sequences and further comprising antigen(s), fragments of the antigen, mixtures of fragments of the antigen, antigens modified to reduce Th2-type immune responses, and fragments of the antigen modified to reduce Th2-type immune responses. Cellular systems for studying immunostimulation by CpG containing nucleic acids include *in vivo*, *in vitro* or *ex vivo* systems.

In an aspect of the present invention, a composition comprising an oligonucleotide comprising a nucleotide sequence 5' purine-purine-C-G-pyrimidine-

pyrimidine 3' wherein C and G are unmethylated and further comprising at least one antigen derived from an allergen that causes anaphylaxis is used to treat or prevent allergies in individuals.

5 In a preferred embodiment, the antigen is derived from the group consisting of food allergens, venom allergens, latex allergens, and drug allergens. In another preferred embodiment, the antigen is a peptide, polypeptide, or protein. In yet another preferred embodiment, the least one antigen comprises a collection of peptides. The collection of peptides can have amino acid sequences that overlap. Alternatively, the collection of peptides can have amino acid sequences that do not overlap. Preferably, 10 the collection of peptides does not have peptides with more than one IgE binding site. If the antigen is a polypeptide, preferably the polypeptide has an amino acid sequence derived from a protein found in foods such as nuts, milk, eggs, seafood and wheat. In addition, peptides, polypeptides and proteins of the present invention can be modified to reduce the binding affinity to IgE antibodies.

15 In another aspect of the invention, a method of treating or preventing allergies and allergies leading to anaphylaxis in individuals is provided. Compositions comprising immunostimulatory oligonucleotides that include unmethylated CpG dinucleotides and also comprising at least one antigen derived from an allergen are administered to individuals who suffer from allergies and allergic reactions that 20 include anaphylaxis. Preferably the allergens are food allergens, venom allergens, latex allergens, and drug allergens. More preferably, the antigens are peptides, polypeptides or proteins although drugs include non-peptide compounds such as penicillin. Preferably, but not necessarily, peptides, polypeptides and proteins of the present invention are modified to reduce the binding affinity to IgE antibodies.

25 In a preferred embodiment of the method, the antigens are collections of peptides having amino acid sequences derived from protein allergens that cause allergies and in some cases, anaphylaxis. The peptides can have overlapping amino acid sequences or the peptides can have amino acid sequences that do not overlap. Preferably, the peptides are synthesized or produce to have no more than a single IgE 30 binding site on each peptide to reduce the ability of the peptides to crosslinking IgE antibodies bound to the surface of mast cells.

In another preferred embodiment of the method, immunostimulatory oligonucleotides include unmethylated CpG dinucleotides in sequence contexts which include 5' TCAACGTT 3', 5' GACGTT 3', 5' AGCGTT 3', 5' AACGCT 3', 5' AACGAT 3', 5' TCAACGTT 3', 5' GACGTT 3', 5' AGACGT 3', 5' AACGCT 3', 5' TGACGTT 3', 5' TAGACGT 3', 5' TAACGCT 3', 5' TCGTCGTTTT 3', and 5' TCGTCGTTTTGTCGTTTTGTCGTT 3'.

In another aspect of the invention, another method of treating or preventing allergies and allergies leading to anaphylaxis in individuals is provided. The method utilizes compositions comprising immunostimulatory oligonucleotides of the present invention conjugated to non-allergenic compound. Without limitation to theory, the conjugation of the oligonucleotide to non-allergic compounds improves the uptake of the nucleic acids by cells related to the immune system such as leukocytes and lymphocytes. The compositions are administered to individuals with allergies to shift an immune response towards a Th1-type immune response. Subsequently or concurrently, compositions comprising antigens, modified antigen and/or collections of antigens are administered to the individuals with Th1 stimulated immune responses.

In a preferred embodiment, individuals with immune systems stimulated Th1-type response can have additional compositions comprising additional antigens, modified antigens and/or collection of antigens administered.

In yet another aspect of the present invention, pharmaceutical compositions comprising immunostimulatory oligonucleotides including unmethylated CpG dinucleotides and at least one antigen derived from allergens that cause allergies and allergies associated with anaphylaxis are provided.

Definitions

“Allergen”: An “allergen” is an antigen that (i) elicits an IgE response in an individual; and/or (ii) elicits an asthmatic reaction (e.g., chronic airway inflammation characterized by eosinophilia, airway hyperresponsiveness, and excess mucus production), whether or not such a reaction includes a detectable IgE response). Preferred allergens for the purpose of the present invention are protein allergens, although the invention is not limited to such. An exemplary list of protein allergens is

presented as an Appendix. This list was adapted on July 22, 1999, from <ftp://biobase.dk/pub/who-iuis/allergen.list>, which provides lists of known allergens.

“Allergic reaction”: An allergic reaction is a clinical response by an individual to an antigen. Symptoms of allergic reactions can affect cutaneous (e.g.,
5 urticaria, angioedema, pruritus), respiratory (e.g., wheezing, coughing, laryngeal edema, rhinorrhea, watery/itching eyes) gastrointestinal (e.g., vomiting, abdominal pain, diarrhea), and/or cardiovascular (if a systemic reaction occurs) systems. For the purposes of the present invention, an asthmatic reaction is considered to be a form of allergic reaction.

10 “Anaphylactic antigen”: An “anaphylactic antigen” according to the present invention is an antigen that is recognized to present a risk of anaphylactic reaction in allergic individuals when encountered in its natural state, under natural conditions. For example, for the purposes of the present invention, pollens and animal danders or excretions (e.g., saliva, urine) are not considered to be anaphylactic antigens. On the
15 other hand, food antigens, insect antigens, drugs, and rubber (e.g., latex) antigens latex are generally considered to be anaphylactic antigens. Food antigens are particularly preferred anaphylactic antigens for use in the practice of the present invention. Particularly interesting anaphylactic antigens are those (e.g., nuts, seeds, seafood, venom) to which reactions are commonly so severe as to create a risk of
20 death.

“Anaphylaxis” or “anaphylactic reaction”, as used herein, refers to an immune response characterized by mast cell degranulation secondary to antigen-induced cross-linking of the high-affinity IgE receptor on mast cells and basophils with subsequent mediator release and the production of pathological responses in
25 target organs, e.g., airway, skin, digestive tract and cardiovascular system. As is known in the art, the severity of an anaphylactic reaction may be monitored, for example, by assaying cutaneous reactions, puffiness around the eyes and mouth, and/or diarrhea, followed by respiratory reactions such as wheezing and labored respiration. The most severe anaphylactic reactions can result in loss of
30 consciousness and/or death.

“Antigen”: An “antigen” is (i) any compound or composition that elicits an immune response; and/or (ii) any compound that binds to a T cell receptor (e.g., when

presented by an MHC molecule) or to an antibody produced by a B-cell. Those of ordinary skill in the art will appreciate that an antigen may be collection of different chemical compounds (e.g., a crude extract or preparation) or a single compound (e.g., a protein). Preferred antigens are protein antigens, but antigens need not be proteins for the practice of the present invention.

“Associated with”: When two entities are “associated with” one another as described herein, they are linked by a direct or indirect covalent or non-covalent interaction. Preferably, the association is covalent. Desirable non-covalent interactions include, for example, hydrogen bonding, van der Waals interaction, hydrophobic interaction, magnetic interaction, etc.

“Fragment”: An antigen “fragment” according to the present invention is any part or portion of the antigen that is smaller than the entire, intact antigen. In preferred embodiments of the invention, the antigen is a protein and the fragment is a peptide.

“IgE binding site”: An IgE binding site is a region of an antigen that is recognized by an anti-antigen IgE molecule. Such a region is necessary and/or sufficient to result in (i) binding of the antigen to IgE; (ii) cross-linking of anti-antigen IgE; (iii) degranulation of mast cells containing surface-bound anti-antigen IgE; and/or (iv) development of allergic symptoms (e.g., histamine release). In general, IgE binding sites are defined for a particular antigen or antigen fragment by exposing that antigen or fragment to serum from allergic individuals (preferably of the species to whom inventive compositions are to be administered). It will be recognized that different individuals may generate IgE that recognize different epitopes on the same antigen. Thus, it is typically desirable to expose antigen or fragment to a representative pool of serum samples. For example, where it is desired that sites recognized by human IgE be identified in a given antigen or fragment, serum is preferably pooled from at least 5-10, preferably at least 15, individuals with demonstrated allergy to the antigen. Those of ordinary skill in the art will be well aware of useful pooling strategy in other contexts.

“Immunodominant”: A particular epitope is considered to be “immunodominant” if it (i) is responsible for a significant fraction of the IgE binding observed with the intact antigen; (ii) is recognized by IgE in a significant fraction of

sensitive individuals; and/or (iii) is a particularly high affinity site. An immunodominant epitope is often defined in reference to the other observed epitopes. For example, all IgE epitopes in a given antigen can be assayed simultaneously (e.g., by immunoblot) and the immunodominant epitopes can be identified by their strength as compared with the other epitopes. Usually, but not always, an immunodominant epitope will contribute at least 10% of the binding reactivity observed in such a study. Alternatively or additionally, an epitope can be classified as immunodominant if it is recognized by IgE in sera of a significant fraction, preferably at least a majority, more preferably at least about 60%, 70%, 80%, 90%, 95%, 99%, or 100%, of sensitive individuals.

“Immunostimulatory oligonucleotide”: “Immunostimulatory oligonucleotides” as used herein are nucleic acid molecules contain unmethylated CpG dinucleotides that stimulate vertebrate lymphocytes and are any length. Preferred immunostimulatory oligonucleotides are between approximately 2 to 100 nucleotides in size. Immunostimulatory oligonucleotides can be single stranded or double stranded. Immunostimulatory oligonucleotides can also contain deoxyribonucleic acids and/or ribonucleic acids. Particularly preferred immunostimulatory oligonucleotides are double-stranded and are approximately 5-50 base pairs in length.

“Mast cell”: As will be apparent from context, the term “mast cell” is often used herein to refer to one or more of mast cells, basophils, and other cells with IgE receptors.

“Oligonucleotide”: An “oligonucleotide” or “oligo” shall mean multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g. adenine (A) or guanine (G)).

“Peptide”: According to the present invention, a “peptide” comprises a string of at least three amino acids linked together by peptide bonds. Inventive peptides preferably contain only natural amino acids, although non-natural amino acids (i.e., compounds that do not occur in nature but that can be incorporated into a polypeptide chain; see, for example, <http://www.cco.caltech.edu/~dadgrp/Unnatstruct.gif>, which displays structures of non-natural amino acids that have been successfully

incorporated into functional ion channels) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in an inventive peptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc.

“Reduced IgE binding”: An inventive composition or antigen fragment is considered to have “reduced IgE binding” if it demonstrates a lower level of interaction with IgE when compared with intact antigen in any available assay. For example, an antigen fragment is considered to have reduced IgE binding if (i) its affinity for anti-antigen IgE (assayed, for example, using direct binding studies or indirect competition studies) is reduced at least about 2-5 fold, preferably at least about 10, 20, 50, or 100 fold as compared with intact antigen; (ii) ability of the fragment to support cross-linking of anti-antigen IgE is reduced at least about 2-fold, preferably at least about 5, 10, 20, 50, or 100 fold as compared with intact antigen; (iii) mast cells containing surface-bound anti-antigen IgE degranulate less (at least about 2 fold, preferably at least about 3, 5, 10, 20, 50, or 100 fold less) when contacted with fragment as compared with intact antigen; and/or (iv) individuals contacted with fragment develop fewer (at least about 2 fold, preferably at least about 3, 5, 10, 20, 50, or 100 fold fewer) allergic symptoms, or developed symptoms are reduced in intensity) when exposed to fragment as compared with intact antigen.

“Sensitized mast cell”: A “sensitized” mast cell is a mast cell that has surface-bound antigen specific IgE molecules. The term is necessarily antigen specific. That is, at any given time, a particular mast cell will be “sensitized” to certain antigens (those that are recognized by the IgE on its surface) but will not be sensitized to other antigens.

“Subject”: A “subject” as used herein is defined as a human or vertebrate animal including without limitation a dog, cat, horse, cow, pig, sheep, goat, chicken, monkey, rat, mouse, rabbit etc.

“Susceptible individual”: According to the present invention, a person is susceptible to a severe and/or anaphylactic allergic reaction if (i) that person has ever displayed symptoms of allergy after exposure to a given antigen; (ii) members of that

person's genetic family have displayed symptoms of allergy against the allergen, particularly if the allergy is known to have a genetic component; and/or (iii) antigen-specific IgE are found in the individual, whether in serum or on mast cells.

“Th1 response” and “Th2 response”: Certain preferred peptides, polypeptides, proteins, immunostimulatory nucleic acids and compositions of the present invention are characterized by their ability to suppress a Th2 response and/or to stimulate a Th1 response preferentially as compared with their ability to stimulate a Th2 response. Th1 and Th2 responses are well-established alternative immune system responses that are characterized by the production of different collections of cytokines and/or cofactors. For example, Th1 responses are generally associated with production of cytokines such as IL-1 β , IL-2, IL-6, IL-12, IL-18, IFN α , IFN γ , TNF β , etc; Th2 responses are generally associated with the production of cytokines such as IL-4, IL-5, IL-10, etc. The extent of T cell subset suppression or stimulation may be determined by any available means including, for example, intra-cytoplasmic cytokine determination. In preferred embodiments of the invention, Th2 suppression is assayed, for example, by quantitation of IL-4, IL-5, and/or IL-13 in stimulated T cell culture supernatant or assessment of T cell intra-cytoplasmic (e.g., by protein staining or analysis of mRNA) IL-4, IL-5, and/or IL-13; Th1 stimulation is assayed, for example, by quantitation of IFN α , IFN γ , IL-2, IL-12, and/or IL-18 in activated T cell culture supernatant or assessment of intra-cytoplasmic levels of these cytokines.

Description of Certain Preferred Embodiments

The present invention provides methods and compositions for modulating an individual's immune response to antigens. It is an aspect of the present invention that allergic responses to antigens, which in some cases lead to asthma and even anaphylaxis, can be treated or prevented by administering compositions having immunostimulatory oligonucleotides including unmethylated CpG sequences. It is another aspect of the present invention that allergies to antigens, especially ones that result in asthma and/or anaphylaxis, can be treated or prevented by administering compositions containing immunostimulatory oligonucleotides having unmethylated CpG dinucleotide sequences and further comprising antigen(s), fragments of the antigen, mixtures of fragments of the antigen, antigens modified to reduce Th2-type

immune responses, and fragments of the antigen modified to reduce Th2-type immune responses. Cellular systems for studying immunostimulation by CpG containing nucleic acids include *in vivo*, *in vitro* or *ex vivo* systems.

5 In general, any oligonucleotide molecule that stimulates an immune response to antigens to elicit a Th1-type immune response may be used in accordance with the present invention. Preferred immunostimulatory oligonucleotides for use in the present invention include any nucleic acid molecules having an unmethylated dinucleotide sequence of CpG. Particularly preferred CpG containing-sequences are palindromic and have the generic sequence of 5' purine-purine-cytosine-guanine-
10 pyrimidine-pyrimidine.

Immunostimulatory Oligonucleotides

Bacterial DNA has been reported to induce the production of cytokines by macrophage and natural killer T-cells (Tokunaga et al. *J. Natl Cancer Inst.* 72:955-
15 962, 1984; Messina et al. *J. Immunol.* 147:1759-1764, 1991). Further studies by Yamamoto et al. on the sequences of bacterial DNA showed that self-complementary sequences within the bacterial DNA were responsible for the immunostimulatory effects (Yamamoto et al. *J. Immunol.* 148:4072-4076, 1992). However, Krieg and coworkers (*Nature* 374:546-549, 1995) were the first to demonstrate that
20 unmethylated CpG sequences within the palindromic sequences were responsible for triggering direct B-cell activation (for a review see Krieg, *Curr. Opin. Immunol.* 12:35-43, 2000. This review article and all cited references therein are incorporated herein by reference).

Unmethylated CpG-containing DNA induce a predominantly Th1 pattern of
25 immune activation characterized by the production of IL-6, IL-12 and IFN- γ in cells. More specifically, oligonucleotides containing CpG repeats are effective in stimulating an immune response when the CpG dinucleotide is unmethylated and present within a particular sequence context. The sequence context that has been reported to be most effective is CpG flanked by two purines on the 5' end and two
30 pyrimidines on the 3' end (5'-Pur-Pur-C-G-Pyr-Pyr-3': see U.S. Patent No. 5,830,877; USP 6,008,200; WO 96/02555, WO 98/18810, WO 98/16247, and WO 98/40100, each of which is incorporated herein by reference). Therefore, immunostimulatory

sequences of the present invention include but are not limited to (5' to 3') AACGCC, AACGCG, AACGGC, AACGGG, ATCGCC, ATCGCG, ATCGGC, ATCGGG, TACGCC, TACGCG, TACGGC, TACGGG, TTCGCC, TTCGCG, TTCGGC, TTCGGG. Preferred immunostimulatory sequences are described by USP 6,008,200 and include 5' TCAACGTT 3', 5' GACGTT 3', 5' AGCGTT 3', 5' AACGCT 3', and 5' AACGAT 3', wherein the CpG dinucleotides are unmethylated. More preferred are immunostimulatory oligonucleotides having the sequences 5' TCAACGTT 3', 5' GACGTT 3', 5' AGACGT 3', or 5' AACGCT 3' with a T nucleotide adjacent to the sequences at the 5' end.

10 Particularly preferred immunostimulatory sequences containing unmethylated CpG dinucleotides which are effective immunostimulants in primate leukocyte cells are described by Krieg and coworkers (*J. Immunol.* 164:1617-24, 2000; incorporated herein by reference). Krieg and coworkers demonstrate that CpG oligonucleotides synthesized with a nuclease-resistant phosphorothioate backbone are potent Th1
15 directed adjuvants in mice. However, these same oligonucleotides are relatively inactive on primate leukocytes in vitro. These results led to experiments that tested over 250 phosphorothioate oligonucleotides with different sequences on their ability to stimulate proliferation and CD86 expression of human B cells and to induce lytic activity and CD69 expression of human NK cells. The experiments testing the over
20 250 sequences demonstrated that sequence, quantity and spacing of individual CpG dinucleotides are factors that affect immunostimulation. Optimally, an oligonucleotide with a TpC dinucleotide at the 5' end juxtaposed to three 6-mer of the sequence (5' GTCGTT 3') separated by TpT dinucleotides stimulated primate leukocytes including humans to a high degree.

25 In addition, Krieg (USP 6,008,200) teaches that CpG-containing oligonucleotides with a modified phosphorothioate backbone are more potent as an immunostimulatory agent than the same oligonucleotides containing phosphodiester backbones. Oligonucleotides containing a nuclease resistant phosphorothioate backbone were approximately 200 times more potent as immunostimulatory nucleic
30 acids than unmodified oligonucleotides as measured by lymphocyte activation using mouse spleen cells. The presence of the phosphorothioate backbone inhibits nuclease digestion which occurs in the serum of subjects treated with immunostimulatory

oligonucleotides or occurs intracellularly. Degradation of the oligonucleotides occurs through digestion by 5' and 3' exonucleases and numerous endonucleases.

Modified Bases and Base Analogs

5 Oligonucleotides for use in accordance with the present invention include any naturally occurring or non-naturally occurring nucleic acids. One of ordinary skill in the art will recognize that many modifications to bases, sugars, and phosphodiester linkages have been made in the literature and that future modifications will also be performed. Nucleic acids for use in accordance with the present invention also
10 include any nucleic acid for use in gene therapy.

 Oligonucleotides of the present invention preferably contain naturally occurring nucleotides. However, modifications to nucleotides, bases, sugars, and phosphodiester backbone that are in accordance with the present invention are also acceptable and in some cases desirable. For example, it has been shown that
15 immunostimulatory oligonucleotides having nuclease resistant phosphorothioate linkages at the ends of the oligos have immunostimulatory effects that are approximately 20 times stronger than oligo that have all naturally occurring phosphodiester linkages.

 Oligonucleotides are polymers of nucleosides joined, generally, through
20 phosphoester linkages. A nucleoside consists of a purine (adenine or guanine or derivative thereof) or pyrimidine (thymine, cytosine or uracil, or derivative thereof) based bonded to a sugar. The four nucleoside units (or bases) in DNA are called deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. A nucleotide is a phosphate ester of a nucleoside.

25 The oligonucleotide of the invention can comprise ribonucleotides (containing ribose as the only or principal sugar component), deoxyribonucleotides (containing deoxyribose as the principal sugar component), or, in accordance with the state of the art, modified sugars or sugar analogs can be incorporated in the immunostimulatory oligonucleotides. Thus, in addition to ribose and deoxyribose, the sugar moiety can
30 be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar can be in pyranosyl or in a furanosyl form. In the immunostimulatory oligonucleotides, the sugar moiety is

preferably the furanoside of ribose, deoxyribose, arabinose or 2'-O-methylribose, and the sugar can be attached to the respective heterocyclic bases either in α or β anomeric configuration. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic
5 base (nucleic acid base) *per se* is known, and need not be described here, except to the extent such preparation can pertain to any specific example.

The phosphorous derivative (or modified phosphate group) which can be attached to the sugar or sugar analog moiety in the oligonucleotides of the present invention can be a monophosphate, diphosphate, triphosphate, alkylphosphate,
10 alkanephosphate, phosphorothioate, phosphorodithioate or the like. A phosphorothioate linkage can be used in place of a phosphodiester linkage. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified nucleotides and oligonucleotides, *per se*, is also known and need not be described here in detail. Peyrottes et al. (1996) *Nucleic Acids Res.*
15 24:1841-1848; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24:2318-2323; and Schultz et al. (1996) *Nucleic Acids Res.* 24:2966-2973. Preferably, oligonucleotides of the present invention comprise phosphorothioate linkages. Oligonucleotides with phosphorothioate backbones can be more immunogenic than those with phosphodiester backbones and appear to be more resistant to degradation after
20 injection into the host. Braun et al. (1988) *J. Immunol.* 141:2084-2089; and Latimer et al. (1995) *Mol. Immunol.* 32:1057-1064.

The heterocyclic bases, or nucleic acid bases, which are incorporated in the immunostimulatory oligonucleotides can be the naturally-occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as
25 mentioned above), as well as naturally-occurring and synthetic modifications of said principal bases.

Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and that as long as other criteria of the
30 present invention are satisfied, the immunostimulatory oligonucleotides can include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. Preferably, however, the heterocyclic base in the

immunostimulatory oligonucleotides includes, but is not limited to, uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the immunostimulatory oligonucleotides via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

In one embodiment, the immunostimulatory oligonucleotides comprises at least one modified base. As used herein, the term "modified base" is synonymous with "base analog", for example, "modified cytosine" is synonymous with "cytosine analog." Similarly, "modified" nucleosides or nucleotides are herein defined as being synonymous with nucleoside or nucleotide "analogs." In a preferred embodiment, a cytosine of the immunostimulatory oligonucleotides is substituted with a cytosine modified by the addition to C-5 and/or C-6 on cytosine with an electron-withdrawing moiety. Preferably, the electron-withdrawing moiety is a halogen. Such modified cytosines can include, but are not limited to, azacytosine, 5-bromocytosine, bromouracil, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, fluorinated cytosine, fluoropyrimidine, fluorouracil, 5,6-dihydrocytosine, halogenated cytosine, halogenated pyrimidine analogue, hydroxyurea, iodouracil, 5-nitrocytosine, uracil, and any other pyrimidine analog or modified pyrimidine.

Synthesis

For use in the present invention, oligonucleotides can be synthesized using any of a number of procedures well known in the art. For example, the β -cyanoethyl phosphoramidite method (S. L. Beaucage and M. H. Caruthers, (1981) *Tet. Let.* 22:1859); nucleoside H-phosphonate method (Garegg et al., (1986) *Tet. Let.* 27: 4051-4054; Froehier et al., (1986) *Nucl. Acid. Res.* 14: 5399-5407; Garegg et al., (1986) *Tet. Let.* 27: 4055-4058. Gaffney et al., (1988) *Tet. Let.* 29:2619-2622). The methods of nucleic acid synthesis can be performed by a variety of automated oligonucleotide synthesizers which are commercially available (for example see PE Biosystems, Foster City, CA).

Alternatively, oligonucleotides can be prepared from existing nucleic acid sequences (e.g. genomic or cDNA) using known techniques, such as those employing restriction enzymes, exonucleases or endonucleases (Ausubel *et al.* "Current Protocols in Molecular Biology". Wiley & Sons: incorporated herein by reference).

5 For use in vivo, oligonucleotides are preferably relatively resistant to degradation by factors and conditions such as acidic conditions, endonucleases and exonucleases. Oligonucleotide stabilization can be accomplished via phosphate backbone modifications. A preferred stabilized oligonucleotide has a phosphorothioate modified backbone. The pharmacokinetics of phosphorothioate oligonucleotides show that they
10 have a systemic half-life of forty-eight hours in rodents and suggest that they may be useful for in vivo applications (Agrawal, S. et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:7595). Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H phosphonate chemistries. Aryl- and alkyl-phosphonates can be made e.g. (as described in U.S. Pat. No. 4,469,863); and
15 alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Pat. No. 5,023,243 and European Patent No. 092,574) can be prepared by automated solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described (Uhlmann, E. and Peyman, A. (1990) *Chem. Rev.* 90:544; Goodchild, J. (1990)
20 *Bioconjugate Chem.* 1:165).

Immunostimulatory oligonucleotides containing unmethylated CpG dinucleotides can be single stranded nucleic acids, or double stranded nucleic acids of any length. Preferred immunostimulatory oligonucleotides are between approximately 2 to 100 nucleotides in size. Immunostimulatory oligonucleotides can also contain
25 deoxyribonucleic acids and/or ribonucleic acids. Particularly preferred immunostimulatory oligonucleotides are double-stranded and are approximately 5-50 base pairs in length. Immunostimulatory oligonucleotides in accordance with the present invention can also be single-stranded or double-stranded circular nucleic acids, preferably DNA.

30

Antigens

In general, inventive compositions comprising immunostimulatory oligonucleotides also comprise immunomodulatory compounds including antigens, cytokines and adjuvants. Preferably, inventive compositions contain antigens, fragments of antigens, a collection of fragments of antigens, antigens modified to reduce binding to IgE, fragments of antigens or modified fragments of antigens that reduce binding to IgE antibodies, fragments of antigens to reduce crosslinking of IgE antibodies bound to mast cells and collections thereof. The term "antigen" is used herein to describe any molecule or compound that elicits an immune response. These antigens include but are not limited to proteins, cytokines, viruses, viral particles, allergens, pollen, dust mite proteins, animal dander, fungal spores, interleukins, interferons, colony stimulating factors, synthetic peptides, polysaccharides, lipids, hormones, naturally occurring molecules with biological activity, and synthetic molecules with biological activity (for example without limitation, enzyme inhibitors, antiviral agents, anticancer agents, anticancer agents). One of ordinary skill in the art will be able to identify antigens for use in the present invention.

In a preferred embodiment, antigens are peptides, polypeptides and proteins preferably having amino acid sequences derived from allergenic proteins found in foods, venom including insect and snake, rubber-base products, animal dander, plant pollen, insect allergens, and/or pharmaceutical drugs. Antigens according to the present invention are provided in compositions comprising immunostimulatory oligonucleotides that include unmethylated CpG sequences. Appendix A presents a representative list of certain known protein antigens. As indicated, the amino acid sequence is known for many or all of these proteins, either through knowledge of the sequence of their cognate genes or through direct knowledge of protein sequence, or both. Of particular interest are anaphylactic antigens.

In a particularly preferred embodiment, the amino acid sequences of antigenic peptides, polypeptides and proteins are derived from food allergens. Non-limiting examples of protein allergens found in food include proteins found in nuts (e.g., peanut, walnut, almond, pecan, cashew, hazelnut, pistachio, pine nut, brazil nut; for reviews see Tariq et al. *British Med. J.* 313:514-517, 1996; Rubira et al. *Allerg Immunol.* 30:212-216, 1998 both incorporated herein by reference), seafood (e.g.

shrimp, crab, lobster, clams), fruit (e.g. plums, peaches, nectarines: *Ann Allergy Asthma Immunol* 7(6):504-8 (1996); cherries, (*Allergy* 51(10):756-7 (1996)), seed antigens(sesame, poppy, mustard), and soy and dairy products (e.g., egg, milk). Some protein allergens found in nuts are related to legume allergies and may be used instead of the legume proteins (e.g. peanuts, soybeans, lentils; *Ann Allergy Asthma Immunol* 77(6): 480-2 (1996)). Also, protein antigens found in pollen-related food allergies may be used (e.g. birch pollen related to apple allergies). Other protein allergens found in foods include those found in young garlic (*Allergy* 54(6):626-9 (1999), and allergens found in snails (*Arch Pediatr* 4(8):767-9 (1997)). Protein allergens in wheat are known to cause exercise-induced allergies (*J Allergy Clin Immunol* 1999 May;103(5 Pt 1):912-7).

In a particularly preferred embodiment, antigens are derived from compounds that cause anaphylactic allergic responses such as food allergies (peanut, milk, egg, wheat),, venoms, rubber-based products such as latex and drugs such as penicillin. Many of these antigens are proteins that cause anaphylactic shock in individuals allergic to the proteins. As non-limiting examples, allergic reactions to nuts, shellfish, and bee venom are known to be severe enough to cause anaphylaxis. (for reviews, see Sicherer, "Manifestations of food allergy: evaluation and management" *Am Fam Physician* 1999 Jan 15;59(2):415-24, 429-30; Anderson, "Milk, eggs and peanuts: food allergies in children." *Am Fam Physician* 1997 Oct 1;56(5):1365-74; Burks and Sampson. "Food allergies in children." *Curr Probl Pediatr* 1993 Jul;23(6):230-52; Heard et al. "Antivenom therapy in the Americas" *Drugs* 1999 Jul;58(1):5-15; Durham and Till. "Immunologic changes associated with allergen immunotherapy" *J Allergy Clin Immunol* 1998;102:157-64). Non-limiting examples of proteins in insect venom include phospholipase A found in bee venom (Weber et al. *Allergy* 42:464-470.). Individuals who are allergic to protein allergens in these compounds develop severe asthma when exposed to these allergens, which triggers IgE crosslinking and release of substances such as histamines and other vasodilators which can lead to anaphylaxis and death.

Modified Antigens

In another preferred embodiment, anaphylactic antigens for use in accordance with the present invention are modified and/or fragmented to remove one or more IgE binding sites. This reduces the ability of the antigen to crosslinking IgE antibodies present on the surface of mast cells. Although some work has described fragments of various inhaled antigens that may have reduced ability to bind and/or cross-link IgE, these are generally not anaphylactic antigens and do not present the same risks to sensitive individuals (see, for example, U.S. Patent No. 5,736,149; U.S. Patent No. 5,891,716; U.S. Patent No. 5,820,862; U.S. Patent No. 5,710,126; U.S. Patent No. 5,591,433; U.S. Patent No. 4,338,297; U.S. Patent No. 4,469,677; U.S. Patent No. 5,648,242; U.S. Patent No. 5,693,495; PCT Patent Application No. WO94/10194; PCT Patent Application No. WO95/34578; PCT Patent Application No. WO99/16467, each of which is incorporated herein by reference). In a particularly preferred embodiment of modified protein antigens. Examples 2-4 provide amino acids sequences of peptides and polypeptides from the peanut protein allergens Ara h 1, 2, and 3 (Burks and Helms, USP 5,558,869; incorporated herein by reference). The IgE bindings sites for these peanut allergens were determined and modified through amino acid deletion, substitution or modification to remove the IgE binding sites (see for example U.S.S.N. 09/141,220 entitled "Methods and Reagents for Decreasing Clinical Reaction to Allergy" filed Aug. 27, 1998 which has been incorporated herein by reference).

Fragment

In another preferred embodiment, the antigen(s) is a peptide or a collection of peptides having amino acid sequences that are derived from protein allergens found in foods, venom, drugs such as penicillin, rubber-based products such as latex, pollen, and insects such as mites and cockroaches. For example, a collection of peptides that represents substantially all of the structural features of a protein antigen is used. In certain particularly preferred embodiments, substantially all of the structural elements of the protein antigen are represented in the peptide collection with substantially all of the peptides having no more than one IgE binding site present within each individual

peptide. Therefore, the ability of the collection of peptides to crosslink IgE molecules bound to the surface of mast cells is reduced or eliminated.

5 In a particularly preferred embodiment of inventive compositions containing peptides or collection of peptides, antigenic compounds are provided in the form of peptide fragments whose amino acid sequences are derived from antigens that cause anaphylaxis in subjects who are allergic to them. An antigen fragment according to the present invention is a portion of the antigen that is smaller than the intact antigen. Inventive compositions including antigen fragments will preferably contain either a sufficiently large number of antigen fragments or at least one antigen fragment that is
10 sufficiently sized that the composition contains one or more immunologically relevant structural element that is present in the intact antigen. For example, certain preferred inventive compositions include at least one of the antigen's T cell epitopes and preferably retain an ability to stimulate T cell proliferation. As mentioned above, the antigen is preferably a protein and the fragment is preferably a peptide. Preferred
15 peptides are at least 6 amino acids long; Particularly preferred peptides are at least about 10, 12, 15, 20, 25, or 30 amino acids long.

In certain embodiments of the invention, peptide antigen fragments have amino acid sequences that are identical to the amino acid sequences of the corresponding portions of the antigen. In certain preferred embodiments of the
20 invention, such peptides having the natural antigen sequence are selected to have reduced IgE binding as compared with the intact antigen by virtue of (i) not including known dominant IgE binding sites; (ii) not including more than one intact IgE binding site; and/or (iii) containing no IgE binding sites.

In other preferred embodiments of the invention, peptide antigen fragments
25 have amino acid sequences that differ from those of the corresponding portions of the antigen in that at least one effective IgE binding site in the intact antigen has been disrupted or removed. Any of a variety of strategies may be employed to disrupt identified IgE binding sites. For example, chemical modifications may be made to amino acids (e.g., to amino acid side chains) within the binding site so as to interfere
30 with its interaction with an IgE molecule. Alternatively or additionally, amino acids may be deleted, inserted, substituted, or stretches of amino acids may be inverted (see, for example, USSN 09/141.220 filed August 27, 1998, entitled "Methods and

Reagents for Decreasing Clinical Reaction to Allergy, incorporated herein. by reference).

Particularly preferred embodiments of the present invention provide compositions comprising multiple antigen fragments. In certain embodiments, the
5 collection of antigen fragments represents substantially all of the primary structural features of the intact antigen (e.g., at least about 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or 99% of the antigen primary structure) .

In other preferred embodiments, the collection represents substantially all such structural features other than one or more that include part or all of an IgE binding
10 site. The strategy of preparing antigen fragment collections that include substantially all of the primary structural features of the intact antigen represents a significant departure from accepted strategies of allergy reduction. In fact, the primary accepted strategy previously has been to select a single fragment, or possibly a small number (fewer than five) of fragments having a selected activity (e.g., T cell stimulation and
15 discarding all other structural information from the antigen (see, for example, U.S. Patents No. 5,820,862; 5,710,126; 5,736,149; 5,480,972; 5/939,283; 5,891,716; 5,843,672; etc.)

For example, Example 3 describes the preparation of a collection of overlapping peptides that represent the entire amino acid sequence of a selected
20 protein antigen; Example 4 describes the use of this collection in an allergy vaccine composition in accordance with the present invention. Those of ordinary skill in the art will recognize that such collections of overlapping peptides may be prepared for any protein antigen. The length of the overlapping peptides is not essential to the invention, though it is generally preferred that the peptides be at least about 6, and
25 more preferably at least about 10, amino acids long. In particularly preferred embodiments, the peptides are at least about 11, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long. Similarly, the extent of overlap is not essential to the present invention, though it is generally preferred that the peptides are offset from one another by no more than about 20, and preferably no more than about 15 amino acids.
30 In particularly preferred inventive compositions, the peptides are offset from one another by no more than about 10, 7, 6, 5, 4, 3, 2, or 1 amino acid.

In certain preferred embodiments, a complete set of overlapping peptides is depleted for certain peptides. For example, peptides that contain dominant IgE binding sites may be removed (see Example 2). Alternatively or additionally, peptides that contain more than one IgE binding site may be removed. In other
5 embodiments of the invention, all peptides containing an intact (or, in some
embodiments, partial) IgE binding site can be removed; or all peptides containing IgE
binding sites of a selected minimum (or maximum) affinity can be removed.

In another preferred embodiment of a collection of peptides, peptides are produced such that individual peptides have only zero or one IgE binding site.
10 Peptides with only zero or one IgE binding site may be produced by limiting the size
of the peptides or by modifying larger peptides and polypeptides with multiple IgE
binding sites to remove one or more of the IgE binding sites. In the absence of
multiple IgE binding sites, peptides can bind to IgE antibodies but are unable to
crosslink IgE antibodies bound to the surface of mast cells to trigger the release of
15 vasodilators such as histamines. Furthermore, a peptide which is limited by size to
only single (or no) IgE binding site, or which is modified to bind to at most only one
IgE antibody, also prevents larger antigens from binding to that IgE antibody to
crosslink to another IgE antibody.

In certain preferred embodiments, it is desirable that the T cell stimulatory
20 capability of the inventive compositions be preserved, and those of ordinary skill in
the art will recognize that the desire to reduce IgE binding capability may be balanced
against the desire to maintain T cell reactivity. Thus, for instance, it will sometimes
be acceptable to leave a particular peptide in an inventive overlapping peptide
composition despite significant IgE binding activity of that peptide if the presence of
25 the peptide also confers significant T cell stimulatory capability, or other desirable
feature, to the composition. Those of ordinary skill in the art will appreciate that it
may be preferred that non-IgE-binding-site elements be preserved to the largest extent
possible, e.g., by selecting overlap sizes and/or end points of fragments so that loss of
information by depletion of IgE binding sites is minimized.

30 In other embodiments, T cells stimulatory capability may or may not be
preserved, but IgE-binding activity without crosslinking IgE molecules preferably is
preserved. Compositions comprising substantially all IgE binding sites of one or

more antigens but lacking IgE crosslinking activity are particularly useful for example for passive immunotherapy treatments. The particular antigen fragment collections described in Example 3 represent groups of like-sized fragments. Such uniformity is not required for the practice of the present invention. Similarly, structural overlap is not required. For example, once IgE binding sites within a given antigen are known, a collection of antigen fragments (presumably of different sizes) can be designed so that each IgE binding site is split onto at least two fragments. In such circumstances, overlap between fragments should generally be minimized or removed. In fact, it may be desirable to create one or more gaps of structural information corresponding to at least part of the IgE binding site.

In some cases, it will be desirable to work in systems in which a single compound (e.g., a single protein) is responsible for most observed allergy. In other cases, the invention can be applied to more complex allergens. Collections of more than one antigen can be used, so that immune responses to multiple antigens may be modulated simultaneously. For peptide fragments, it will often be desirable to include fragments from multiple different antigens in the composition of the present invention. To give but one example, at least three different proteins, Ara h 1, Ara h 2, and Ara h 3, are thought to contribute to peanut allergy; >90% of individuals who are allergic to peanuts have IgE reactive with Ara h 1, >90 % of allergic individuals have IgE reactive with Ara h 2, and >44% have IgE reactive with Ara h 3. Inventive compositions may include fragments from more than one of these proteins, from all of them, or from all of them plus additional peanut proteins. Also, it may be desirable to include fragments of a variety of different kinds of antigens so that multiple allergies are treated simultaneously.

Inventive peptide antigen fragments may be produced by any available method including but not limited to chemical or proteolytic cleavage of intact antigen, chemical synthesis, or *in vitro* or *in vivo* expression of an isolated or recombinant nucleic acid molecule. As can be seen with reference to the Appendix, a large number of genes for protein antigens have been cloned and are available for manipulation. In certain preferred embodiments, inventive peptides are prepared by chemical synthesis. Such peptides may utilize only naturally-occurring amino acids.

or may include one or more non-natural amino acid analog or other chemical compound capable of being incorporated into a peptide chain.

Any of a variety of compositions comprising the inventive antigen fragments may be utilized in the practice of the present invention. For example, one or more
5 chemical groups may be linked to the antigen fragment (e.g., a carbohydrate moiety may be linked to an amino acid). Alternatively or additionally, inventive peptides may be produced as a fusion with another polypeptide chain. In some embodiments, it may be desirable to include a cleavage site within such a fusion peptide, that can be activated by an enzyme, a chemical, or by experimental conditions (e.g., pH).

10 Inventive antigen and antigen fragments may be provided in pure form, or may be crude preparations, such as a chemical or proteolytic digestion of a food extract (see, for example, Hong et al. *J. Allergy Clin. Immunol.* 104:473, August 1999). Those of ordinary skill in the art will appreciate that any preparation or formulation of antigen and antigen fragments may be employed in the practice of the present
15 invention. Additionally, inventive fragments may be provided by combination or association with one or more other agents, as discussed in more detail below.

The amount of antigen to be employed in any particular composition or application will depend on the nature of the particular antigen and of the application for which it is being used, as will readily be appreciated by those of ordinary skill in
20 the art. In general, larger amounts of antigen are useful for inducing Th1 responses, smaller amounts for inducing Th2 responses.

Conjugation

The present invention provides immunomodulatory compositions containing
25 immunostimulatory oligonucleotides and antigens as described by the present teachings. Oligonucleotides and antigens are provided in compositions with or without conjugation where conjugation is defined as association through covalent, electrostatic, or magnetic interactions. It is recognized that CpG containing oligonucleotides can be conjugated to antigenic compounds through high affinity
30 interactions or covalent bonds. In addition, immunostimulatory oligonucleotides can be admixed with antigenic or modified antigenic compounds in accordance with the

present teachings and administered to subjects and individuals in compositions without conjugation or association.

5 In certain preferred embodiments, the immunostimulatory oligonucleotides and antigens are conjugated. Conjugation of immunostimulatory oligonucleotides and antigens is optional, however. Certain preferred collections of antigenic peptides according to the present invention may be conjugated to immunostimulatory oligonucleotides or may be provided in compositions with immunostimulatory oligonucleotides without conjugation. For compositions comprising immunostimulatory oligonucleotides and mixtures of peptides, it may be preferable
10 not to conjugate the peptides with the oligonucleotides.

For methods of conjugation, WO98/55495 (Schwartz et al.) teaches the conjugation of CpG containing immunostimulatory oligonucleotides to antigenic molecules. These teachings are incorporated herein by reference. The immunostimulatory oligonucleotides portion can be coupled with the
15 immunomodulatory molecule portion of a conjugate in a variety of ways, including covalent and/or non-covalent interactions.

The link between the portions can be made at the 3' or 5' end of the immunostimulatory oligonucleotide, or at a suitably modified base at an internal position in the immunostimulatory oligonucleotide. If the immunomodulatory
20 molecule is a peptide and contains a suitable reactive group (e.g., an N-hydroxysuccinimide ester) it can be reacted directly with the N⁴ amino group of cytosine residues. Depending on the number and location of cytosine residues in the immunostimulatory oligonucleotide, specific labeling at one or more residues can be achieved.

25 Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the immunostimulatory oligonucleotide. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, the immunomodulatory molecule of interest.

30 Where the immunomodulatory molecule is a peptide, this portion of the conjugate can be attached to the 3'-end of the immunostimulatory oligonucleotide through solid support chemistry. For example, the immunostimulatory

oligonucleotide portion can be added to a polypeptide portion that has been pre-synthesized on a support. Haralambidis et al. (1990a) *Nucleic Acids Res.* 18:493-499; and Haralambidis et al (1990b) *Nucleic Acids Res.* 18:501-505. Alternatively, the immunostimulatory oligonucleotide can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end. Upon chemical cleavage of the immunostimulatory oligonucleotide from the support, a terminal thiol group is left at the 3'-end of the oligonucleotide (Zuckermann et al. (1987) *Nucleic Acids Res.* 15:5305-5321; and Corey et al. (1987) *Science* 238:1401-1403) or a terminal amine group is left at the 3'-end of the oligonucleotide (Nelson et al. (1989) *Nucleic Acids Res.* 17:1781-1794). Conjugation of the amino-modified immunostimulatory oligonucleotides to amino groups of the peptide can be performed as described in Benoit et al. (1987) *Neuromethods* 6:43-72. Conjugation of the thiol-modified immunostimulatory oligonucleotides to carboxyl groups of the peptide can be performed as described in Sinah et al. (1991) *Oligonucleotide Analogues: A Practical Approach*, IRL Press. Coupling of an oligonucleotide carrying an appended maleimide to the thiol side chain of a cysteine residue of a peptide has also been described. Tung et al. (1991) *Bioconjug. Chem.* 2:464-465.

The peptide portion of the conjugate can be attached to the 5'-end of the immunostimulatory oligonucleotides through an amine, thiol, or carboxyl group that has been incorporated into the oligonucleotide during its synthesis. Preferably, while the oligonucleotide is fixed to the solid support, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite at the other, is covalently attached to the 5'-hydroxyl. Agrawal et al. (1986) *Nucleic Acids Res.* 14:6227-6245; Connolly (1985) *Nucleic Acids Res.* 13:4485-4502; Kremsky et al. (1987) *Nucleic Acids Res.* 15:2891-2909; Connolly (1987) *Nucleic Acids Res.* 15:3131-3139; Bischoff et al (1987) *Anal. Biochem.* 164:336-344; Blanks et al. (1988) *Nucleic Acids Res.* 16:10283-10299; and U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802. Subsequent to deprotection, the latent amine, thiol, and carboxyl functionalities can be used to covalently attach the oligonucleotide to a peptide. Benoit et al. (1987); and Sinah et al. (1991).

The peptide portion can be attached to a modified cytosine or uracil at any position in the immunostimulatory oligonucleotide. The incorporation of a "linker

arm" possession a latent reactive functionality, such as an amine or carboxyl group. at C-5 of the modified base provides a handle for the peptide linkage. Ruth, *4th Annual Congress for Recombinant DNA Research*, p. 123.

5 An immunostimulatory oligonucleotide-immunomodulatory molecule conjugate can also be formed through non-covalent interactions, such as ionic bonds, hydrophobic interactions, hydrogen bonds and/or van der Waals attractions. Non-covalently linked conjugates can include a non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an immunostimulatory oligonucleotide. Roget et al. (1989) *Nucleic Acids*
10 *Res.* 17:7643-7651. Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated oligonucleotide.

Non-covalent associations can also occur through ionic interactions involving an immunostimulatory oligonucleotide and residues within the immunomodulatory
15 molecule, such as charged amino acids, or through the use of a linker portion comprising charged residues that can interact with both the oligonucleotide and the immunomodulatory molecule. For example, non-covalent conjugation can occur between a generally negatively-charged immunostimulatory oligonucleotides and positively-charged amino acid residues of a peptide. e.g., polylysine and polyarginine
20 residues.

An antigen can also be conjugated to immunostimulatory oligonucleotides nucleic acids by covalently or electrostatically coupling the antigen to any molecule that can bind to nucleic acids with high affinity (high affinity is defined herein as having an association constant greater than 10^6). Non-covalent conjugation between
25 immunostimulatory oligonucleotides and immunomodulatory molecules can occur through DNA binding motifs of molecules that interact with DNA as their natural ligands. For example, such DNA binding motifs can be found in transcription factors and anti-DNA antibodies. Preferably, the antigen is covalently linked to a polypeptide containing a nucleic acid binding domain. Most preferably, the nucleic
30 acid binding domain is a sequence-specific DNA binding domain. Synthesis of fusion proteins containing an antigen and nucleic acid binding domain may be performed using recombinant DNA technology well known to one of ordinary skill in the art.

Coupling of non-peptide antigens to polypeptides can be performed by chemical reactions known to those of ordinary skill in the protein chemistry art (Coligan, *et al.* "Current Protocols in Protein Science." John Wiley & Sons; incorporated herein by reference.).

5 Non-limiting examples of molecule-nucleic acid interactions include protein-DNA (Pabo CO, *et al.* "Protein-DNA recognition." *Annu Rev Biochem.* 1984. 53:293-321), protein-RNA (Draper. "Protein-RNA recognition." *Annu Rev Biochem.* 1995;64:593-620.), single-stranded oligonucleotides bound to single-stranded oligonucleotides, minor groove binding drugs bound to double stranded DNA/RNA
10 (Wemmer, *et al.* "Targeting the minor groove of DNA." *Curr Opin Struct Biol.* 1997. 7(3):355-61; Waring. "Binding of antibiotics to DNA." *Ciba Found Symp.* 1991. 158:128-42. discussion 142-6, 204-12), major groove binding molecules bound to double stranded DNA or RNA, base pair intercalators bound to nucleic acids (Baguley. "DNA intercalating anti-tumor agents." *Anticancer Drug Des.* 1991.
15 6(1):1-35), negatively charged phosphate binders, and metals complexes that bind to DNA (Stemp, *et al.* "Electron transfer between metal complexes bound to DNA: is DNA a wire?" *Met Ions Biol Syst.* 1996. 33:325-65; all references in the preceding paragraph are incorporated herein by reference.)

Other non-limiting examples of nucleic acid structures that can bind molecules
20 include G-quartets, telomeres, circular DNA and RNA (single-stranded and double-stranded), holliday junctions, cube-like structures (Seeman. "DNA nanotechnology: novel DNA constructions." *Annu Rev Biophys Biomol Struct.* 1998. 27:225-48). RNA secondary structure, and triple-stranded nucleic acids.

For conjugation of immunostimulatory oligonucleotides with antigens through
25 DNA-protein interactions, the nucleic acid component containing the immunostimulatory oligonucleotide also contains sequences or higher order structures that serve as binding sites for the proteins coupled to antigens. A preferred binding site is a sequence-specific nucleic acid binding site for a polypeptide. Preferably, the binding sites are not within the immunostimulatory oligonucleotide. Preferably, the
30 binding between the antigen-containing component and the immunostimulatory oligonucleotide-containing component is electrostatic or magnetic.

In a preferred embodiment, the antigens are coupled to immunostimulatory oligonucleotides via electrostatic protein-nucleic acid interactions. The composition designed to modulate an immune response contains at least four components. The immunostimulatory oligonucleotide and the antigen constitute two components which
5 are brought into association by the third and fourth components. The third component is a nucleic acid sequence, preferably covalently linked to the immunostimulatory oligonucleotide, that contains a sequence for binding to the fourth component. The nucleic acid of the third component can be DNA or RNA or derivatives thereof known to those of ordinary skill in the art and familiar with the prior art. The nucleic
10 acid of the third component can be single-stranded or double-stranded.

The fourth component is any molecule that can bind to the nucleic acid of the third component by electrostatic or magnetic interactions with high affinity. Preferably, the fourth component is a polypeptide containing a sequence-specific DNA-binding domain. Many sequence-specific DNA-binding domains used in
15 fusions proteins to target polypeptides to specific DNA sequences are well known in the art (Two-hybrid technology, Ausubel *et al.* "Current Protocols in Molecular Biology". Wiley & Sons: Numerous reviews on protein-DNA interactions exist. A few are listed for incorporation by reference. Pabo CO, *et al.* "Transcription factors: structural families and principles of DNA recognition." *Annu Rev Biochem.* 1992.
20 61:1053-95; Pabo CO, *et al.* "Protein-DNA recognition." *Annu Rev Biochem.* 1984. 53:293-321; Berg JM. *et al.* "Lessons from zinc-binding peptides." *Annu Rev Biophys Biomol Struct.* 1997. 26:357-71.)

In another preferred embodiment of the present invention, a composition comprises an antigen-containing component and an immunostimulatory
25 oligonucleotide-containing component, where the association between the two components is electrostatic or magnetic. More specifically, the interaction between the two components is mediated through protein-nucleic acid interactions wherein the protein is a fusion protein comprising the antigen and a nucleic acid binding domain. Preferably, the fusion protein comprises the antigen and sequence-specific DNA
30 binding domain. If the antigen is not a polypeptide, the nucleic acid-binding domain can be linked to the antigen covalently, or through electrostatic or magnetic interactions. Alternatively but without limitation, the polypeptide comprising the

nucleic acid binding domain can be conjugated with the antigen through non-covalent electrostatic interactions such as a biotin-streptavidin association.

The linkage of the immunostimulatory oligonucleotide to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugated (Yanagawa et al. (1988) *Nucleic Acids Symp. Ser.* 19:189-192), oligonucleotide-fatty acid conjugates (Grabarek et al. (1990) *Anal. Biochem.* 185:131-135; and Staros et al. (1986) *Anal. Biochem.* 156:220-222), and oligonucleotide-sterol conjugates. Boujrad et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5728-5731.

The linkage of the oligonucleotide to an oligosaccharide can be formed using standard known methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin. O'Shannessy et al. (1985) *J. Applied Biochem.* 7:347-355.

The linkage of a circular immunostimulatory oligonucleotides to a peptide or antigen can be formed in several ways. Where the circular immunostimulatory oligonucleotide is a synthesized using recombinant or chemical methods, a modified nucleoside is suitable. Ruth (1991) in *Oligonucleotides and Analogues: A Practical Approach*, IRL Press. Standard linking technology can then be used to connect the circular immunostimulatory oligonucleotide to the antigen or other peptide. Goodchild (1990) *Bioconjug. Chem.* 1:165. Where the circular immunostimulatory oligonucleotide is isolated, or synthesized using recombinant or chemical methods, the linkage can be formed by chemically activating, or photoactivating, a reactive group (e.g. carbene, radical) that has been incorporated into the antigen or other peptide.

Additional methods for the attachment of peptides and other molecules to oligonucleotides can be found in U.S. Patent No. 5,391,723; Kessler (1992) "Nonradioactive labeling methods for nucleic acids" in Kricka (ed.) *Nonisotopic DNA Probe Techniques*. Academic Press; and Geoghegan et al. (1992) *Bioconjug. Chem.* 3:138-146.

Oligonucleotides can be ionically, or covalently associated with appropriate molecules using techniques which are well known in the art. A variety of coupling or

crosslinking agents can be used e.g. protein A, carbodiimide, and N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP).

Non-antigenic carrier

5 In another preferred embodiment, inventive compositions comprise immunostimulatory oligonucleotides and a substantially non-allergenic compound. Alternatively, the substantially non-allergenic compound is a compound with low allergenicity. Preferably, the compound is a peptide, polypeptide or protein which can facilitate the cellular uptake of the immunostimulatory oligonucleotides. Non-
10 limiting examples of non-allergenic proteins are proteins derived from the same or similar organism. For human subjects, proteins such as human serum albumin or proteins from subjects are less likely to be allergenic than proteins from other subjects or organisms. Therefore, these proteins are used in accordance with the present invention to improve the uptake of immunostimulatory oligonucleotides without
15 utilizing antigenic compounds.

 Krieg et al. (*Antisense Nucleic Acid Drug Dev.* 1998 8:351-356) demonstrated that CpG containing oligonucleotides mixed with DNA vaccines did not improve the efficacy of the vaccine and speculated that uptake of the phosphorothioate backbone CpG oligonucleotide interfered with the uptake and expression of the plasmid (Krieg.
20 *Curr. Opin. Immunol.* 12:35-43, 2000). CpG oligonucleotides conjugated to non-allergenic compounds or low allergenic compounds would improve the uptake of the immunostimulatory oligonucleotide and stimulate the immune system. Concurrent or subsequent to administration to cells or subjects of immunostimulatory oligonucleotides conjugated to non-allergenic proteins or proteins with low
25 antigenicity, antigens according to the present teachings are then administered to cells or subjects stimulated by the immunostimulatory oligonucleotide-conjugate.

 Therefore, inventive methods of the present invention include treating an individual first with a composition containing CpG oligonucleotides preferably but not necessarily conjugated to a non-allergenic compound such as a protein isolated
30 from that individual. Subsequent to, or concurrently with, administration of a CpG oligonucleotide composition, a second composition containing antigenic compounds (peptides, polypeptides, proteins, modified proteins according to the present

5 teachings) is administered to the individual. Thus using this method, the immune response of an individual or subject is first stimulated to have a Th1-type response using the CpG containing composition before administration of the antigenic compound. This two-step method of vaccination activates an individual's immune system towards production of IgG and IgM antibodies and downregulates the production of IgE antibodies associated with allergic and possibly anaphylactic responses.

10 In addition, in certain embodiments where the CpG-containing immunostimulatory oligonucleotides are conjugated to generally non-allergenic compounds, the conjugates can be used for treating multiple subjects and individuals with different allergic conditions. Thus, the conjugation of the immunostimulatory oligonucleotides to a generally non-allergenic compound increases the number of individuals that can be treated.

15 Furthermore, the immunostimulatory oligonucleotide and the antigen and/or immunomodulatory facilitator can be administered together in the form of a conjugate or co-administered in an admixture sufficiently close in time so as to modulate an immune response. Preferably, the immunostimulatory oligonucleotide and immunomodulatory molecule are administered simultaneously. The term "co-administration" as used herein refers to the administration of at least two different substances sufficiently close in time to modulate an immune response. Preferably, 20 co-administration refers to simultaneous administration of at least two different substances.

Bacterial Delivery

25 In another preferred embodiment, immunostimulatory nucleic acids and antigens are delivered to subjects or individuals using microorganisms. Any microorganism capable of producing immunomodulatory peptides, polypeptides, or proteins can be used in accordance with the present invention. Preferably, the microorganism is bacterial and gram-negative. However, gram-positive bacteria may also be used in the present invention. Non-limiting examples of bacteria include *E. coli*, *Salmonella*, *Listeria*, *Mycobacterium*, *Legionella*, *Vibrio cholera*, *Shigella*, 30 *Yersenia*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*.

Serratia, *Plesiomonas*, *Aeromonas*. Particularly preferred attenuated microorganisms are infectious intracellular pathogens which are phagocytosed by antigen-presenting cells in individuals who are exposed to the microorganism. Examples of microorganisms which are intracellular pathogens include *Salmonella*,

5 *Mycobacterium*, *Leishmania*, *Legionella*, *Listeria*, and *Shigella*.

Preferably, microorganisms are bacteria which are genetically engineered to produce antigenic peptides, polypeptides or proteins according to the teachings of the present invention. More preferably, bacteria are killed (preferably heat-killed) or attenuated (i.e., non-toxic strain) and administered to individuals in suitable
10 encapsulation materials and/or as pharmaceutical compositions for vaccines.

Several bacterial systems for use as delivery vehicles are known. For example, Calderwood *et al.* (US Patent 5,747,028) utilize *Vibrio cholerae* as a delivery vehicle for production of heterologous antigens for use as a live vaccine against infectious organisms. Miller and Mekalanos (US Patent 5,731,196) utilize
15 *Salmonella* as delivery vehicle for production of heterologous antigens for use as a live vaccine against infectious organisms. Hess *et al.* (PNAS 93:1458-1463, 1996) utilize recombinant attenuated *Salmonella* which secretes antigenic determinants of *Listeria* as a live vaccine to protect against listeriosis.

As previously described, bacterial DNA has been shown to have
20 immunostimulatory effects (Yamamoto *et al.* *J. Immunol.* 148:4072-4076, 1992). Therefore, administration of compositions to subjects comprising genetically engineered bacteria which produce desired peptides, polypeptides, or proteins having amino acid sequences derived from allergenic antigens would provide immunostimulatory effects without using additional CpG-containing oligonucleotides.
25 However, it is recognized that additional CpG-containing oligonucleotides with immunostimulatory effects (for example, see *J. Immunol.* 164:1617-24, 2000) may be included in compositions with antigen-producing bacteria.

Microorganisms that can be genetically manipulated to produce a desired polypeptide by one skilled in the art are preferred (Ausubel *et al.* *Current Protocols in*
30 *Molecular Biology*, Wiley and Sons, Inc. 1999, incorporated herein by reference). Genetic manipulation includes mutation of the host genome, insertion of genetic material into the host genome, deletion of genetic material of the host genome.

transformation of the host with extrachromosomal genetic material. transformation with linear plasmids, transformation with circular plasmids. insertion of genetic material into the host (i.e injection of mRNA), insertion of transposons. and chemical modification of genetic material. Techniques for generating nucleic acids including an expressible gene. and for introducing such nucleic acids into an expression system in which any protein encoded by the expressible gene will be produced. are well established in the art (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, incorporated herein by reference).

Preferably, the microorganism is genetically engineered to produce a desired level (preferably high level) of antigenic peptides, polypeptides, or proteins and heat-killed before administration to subjects in a suitable pharmaceutical composition as a vaccine. As a non-limiting example of a suitable microorganism, heat-killed *Listeria monocytogenes* is an effective adjuvant which downregulates the Th2-type immune response and upregulates the Th1-type immune response (Yeung et al. *J. Immunol.* 161:4146-4152, 1998). *Listeria* is also a bacterium that can be genetically manipulated to produced desired peptides and polypeptides. Therefore, *Listeria* is a suitable bacterium to produce peptide, polypeptide and protein antigens and is also suitable as an adjuvant for delivery to subjects.

Polypeptide production by bacteria can be accomplished without secretion of the polypeptide. An advantage of non-secreted polypeptide antigens is the protection afforded by the cellular membrane during a process used to kill bacteria before administration to subjects. A preferred process of killing is heat-killing. However, any method of killing bacteria which does not degrade genetically engineered peptides, or polypeptides and hence destroy any desired physical or immunological properties of the peptides and polypeptides may be used. In addition, production of polypeptides by bacteria that are highly allergenic or anaphylactic without secretion by bacteria decreases exposure of the allergens to IgE molecules when administered to subjects. As a result, the polypeptides within bacteria are not released until after phagocytosis and digestion of the polypeptides by antigen-presenting cells. It is recognized that using live attenuated bacteria to produce non-secreted polypeptides

also decreases exposure of the polypeptides to IgE molecules after administration to subjects.

5 Bacteria that produce and secrete inventive peptides and polypeptides according to the present teachings can also used to deliver antigenic polypeptides to subjects. A variety of bacterial secretion signals that can be used in the practice of the present invention are known. The Sec-dependent process in *E. coli* is one which is well known (for a review see Driessen et al. *Curr. Opin. Microbiology* 1:216-22). In addition, the OmpA signal peptide in *E. coli* has been described by Wong and Sutherland (US Patent 5.223.407). Fusion proteins containing either of these
10 secretion signal peptides are not fully secreted by the bacteria, but rather only transported across the inner membrane of the gram-negative bacteria into the periplasm. These secretion signals may be used in the present invention to transport immunomodulatory polypeptides into the periplasm of bacteria. After administration of the genetically engineered bacteria to an individual and subsequent phagocytosis by APCs, the immunomodulatory polypeptides in the periplasm are released after
15 degradation of the outer membrane of the bacteria in the endosome. Release of the polypeptides inside the APC's therefore reduces exposure of allergenic polypeptides to IgE antibodies bound to mast cells. Bacteria that secrete inventive polypeptides into the periplasm for use in accordance with the present invention can be killed
20 (preferably heat-killed) before administration to subjects. Bacteria that secrete inventive polypeptides into the periplasm for use in accordance with the present invention can also administered to subjects as live attenuated organisms.

Adjuvants

25 In certain preferred embodiments of the invention, the inventive compositions are provided with one or more immune system adjuvants other than immunostimulatory oligonucleotides. A large number of adjuvant compounds is known; a useful compendium of many such compounds is prepared by the National Institutes of Health and can be found on the world wide web
30 (<http://www.niaid.nih.gov/daids/vaccine/pdt/compendium/pdf>, incorporated herein by reference; see also Allison *Dev. Biol. Stand.* 92:3-11, 1998; Unkeless et al. *Annu. Rev. Immunol.* 6:251-281, 1998; and Phillips et al. *Vaccine* 10:151-158, 1992, each of

which is incorporated herein by reference). Preferred adjuvants are characterized by an ability to stimulate a Th1 responses preferentially over Th2 responses and/or to down regulate Th2 responses. In fact, in certain preferred embodiments of the invention, adjuvants that are known to stimulate Th2 responses are avoided.

5 Particularly preferred adjuvants include, for example, preparations (including heat-killed samples, extracts, partially purified isolates, or any other preparation of a microorganism or macroorganism component sufficient to display adjuvant activity) of microorganisms such as *Listeria monocytogenes* or others (e.g., Bacille Calmette-Guerin [BCG], *Corynebacterium* species, *Mycobacterium* species, *Rhodococcus*
10 species, *Eubacteria* species, *Bordetella* species, and *Nocardia* species), and preparations of nucleic acids that include unmethylated CpG motifs (see, for example, U.S. Patent No. 5,830,877; and published PCT applications WO 96/02555, WO 98/18810, WO 98/16247, and WO 98/40100, each of which is incorporated herein by reference). Other preferred adjuvants reported to induce Th1-type responses and not
15 Th2-type responses include, for example, Aviridine (N,N-dioctadecyl-N'N'-bis (2-hydroxyethyl) propanediamine) and CRL 1005.

In some embodiments of the invention, the adjuvant is associated (covalently or non-covalently, directly or indirectly) with the antigen so that adjuvant and antigen can be delivered substantially simultaneously to the individual, optionally in the
20 context of a single composition. In other embodiments, the adjuvant is provided separately. Separate adjuvants may be administered prior to, simultaneously with, or subsequent to administration of inventive antigenic compositions. In certain preferred embodiments of the invention, a separate adjuvant composition is provided that can be utilized with multiple different antigenic compositions.

25 Where adjuvant and inventive compositions are provided together, any association sufficient to achieve the desired immunomodulatory effects may be employed. Those of ordinary skill in the art will appreciate that covalent associations will sometimes be preferred. For example, where adjuvant and inventive peptides, polypeptides or proteins are both polypeptides, a fusion polypeptide may be
30 employed.

Targeting

Inventive compositions may desirably be associated with a targeting entity that will ensure their delivery to a particular desired location. In preferred embodiments of the invention, inventive compositions are targeted for uptake by antigen presenting cells. For example, inventive compositions could be targeted to dendritic cells or macrophages via association with a ligand that interacts with an uptake receptor such as the mannose receptor or an Fc receptor. Inventive compositions comprising antigens or modified antigens conjugated to immunostimulatory oligonucleotides could be targeted to other APCs via association with a ligand that interacts with the complement receptor. Inventive compositions comprising antigens or modified antigens could be specifically directed to dendritic cells through association with a ligand for DEC205, a mannose-like receptor that is specific for these cells.

Alternatively or additionally, inventive compositions comprising antigens or modified antigens conjugated to immunostimulatory oligonucleotides could be targeted to particular vesicles within APCs. Those of ordinary skill in the art will appreciate that any targeting strategy should allow for proper uptake and processing of antigen by the APCs.

Immunostimulatory compositions of the present invention can be targeted by association of the antigen-containing composition with an Ig molecule, or portion thereof. Ig molecules are comprised of four polypeptide chains, two identical "heavy" chains and two identical "light" chains. Each chain contains an amino-terminal variable region, and a carboxy-terminal constant region. The four variable regions together comprise the "variable domain" of the antibody; the constant regions comprise the "constant domain". The chains associate with one another in a Y-structure in which each short Y arm is formed by interaction of an entire light chain with the variable region and part of the constant region of one heavy chain, and the Y stem is formed by interaction of the two heavy chain constant regions with one another. The heavy chain constant regions determine the class of the antibody molecule, and mediate the molecule's interactions with class-specific receptors on certain target cells; the variable regions determine the molecule's specificity and affinity for a particular antigen.

Class-specific antibody receptors, with which the heavy chain constant regions interact, are found on a variety of different cell types and are particularly concentrated on professional antigen presenting cells (pAPCs), including dendritic cells. According to the present invention, inventive compositions may be targeted for delivery to pAPCs through association with an Ig constant domain. In one embodiment, an Ig molecule is isolated whose variable domain displays specific affinity for the antigen to be delivered, and the antigen is delivered in association with the Ig molecule. The Ig may be of any class for which there is an Ig receptor, but in certain preferred embodiments, is an IgG. Also, it is not required that the entire Ig be utilized; any piece including a sufficient portion of the Ig heavy chain constant domain is sufficient. Thus, Fc fragments and single-chain antibodies may be employed in the practice of the present invention.

In one embodiment of the invention, inventive antigenic compositions are prepared as a fusion molecule of antigens or modified antigens with at least an Ig heavy chain constant region (e.g., with an Fc fragment), so that a single polypeptide chain, containing both antigen and Ig heavy chain constant region components, is delivered to the individual (or system). It is recognized that immunostimulatory oligonucleotides may be conjugated to the fusion molecule. This embodiment allows increased flexibility of antigen selection because the length and character of the antigen is not constrained by the binding requirements of the Ig variable domain cleft. In particularly preferred versions of this embodiment, the antigenic composition and the Fc portion of the fusion molecule are separated from one another by a severable linker that becomes cleaved when the fusion molecule is taken up into the pAPC. A wide variety of such linkers is known in the art. Fc fragments may be prepared by any available technique including, for example, recombinant expression (which may include expression of a fusion protein) proteolytic or chemical cleavage of Ig molecules (e.g., with papain), chemical synthesis, etc.

30 **Pharmaceutical Compositions**

Compositions of the present invention can be administered to a host via pharmaceutically acceptable compositions. Non-aqueous solvents include propylene

glycol, polyethylene glycol, vegetable oils, and ethyl oleate. Aqueous carriers include water, saline, alcoholic/aqueous solutions, emulsions or suspensions or buffered solutions. Compositions for parenteral delivery include sodium chloride solutions, Ringer's dextrose, dextrose and NaCl solutions, lactated Ringer's or fixed oils.

5 Compositions for intravenous delivery include fluid and nutrient replenishers, electrolyte replenishers. Preservatives and other additives may also be utilized. These include antimicrobial agents, antioxidants, chelating agents, and inert gases.

Adsorption promoters for nasal drug delivery, mucosal drug delivery and skin drug delivery can facilitate adsorption of compositions into target tissues. One of

10 ordinary skill in the art may consult Chien, "Novel Drug Delivery Systems," Marcel Dekker, 1992, for further details (see also Sloan, "Prodrugs: topical and Ocular Drug Delivery," Marcel Dekker, 1992; both incorporated herein by reference). Those of ordinary skill in the clinical arts will be familiar with or can readily ascertain, means for drug delivery into skin and mucosa. For intranasal drug delivery vehicles and

15 systems see Chien, Novel Drug Delivery Systems, Chapter 5, Marcel Dekker, 1992 (incorporated herein by reference). For dermal routes of administration and subcutaneous injections, also see Chien, Novel Drug Delivery Systems, Chapter 7, Marcel Dekker, 1992, (incorporated herein by reference).

Pharmaceutical compositions for use in accordance with the present invention

20 may include a pharmaceutically acceptable excipient or carrier. As used herein, the term "pharmaceutically acceptable carrier" means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Some examples of materials which can serve as pharmaceutically acceptable carriers are sugars such as lactose, glucose, and sucrose; starches such as corn starch and

25 potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols: such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering

30 agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium

lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator. The pharmaceutical compositions of this invention can be administered
5 to humans and/or to other animals, orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, topically (as by powders, ointments, or drops), buccally, or as an oral or nasal spray.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In
10 addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive,
15 castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Injectable preparations, for example, sterile injectable aqueous or oleaginous
20 suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution,
25 U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables.

The injectable formulations can be sterilized, for example, by filtration
30 through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

In order to prolong the effect of an agent, it is often desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the agent then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of agent to polymer and the nature of the particular polymer employed, the rate of release of the agent can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides) Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the compounds of this invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active compound.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active compound is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid

polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

5 Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active
10 ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well
15 as high molecular weight polyethylene glycols and the like.

The compounds can also be in micro-encapsulated form with one or more excipients as noted above. The solid dosage forms of tablets, dragees, capsules, pills and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the pharmaceutical formulating
20 art. In such solid dosage forms the active compound may be admixed with at least one inert diluent such as sucrose, lactose or starch. Such dosage forms may also comprise, as is normal practice, additional substances other than inert diluents, e.g., tableting lubricants and other tableting aids such as magnesium stearate and microcrystalline cellulose. In the case of capsules, tablets and pills, the dosage forms
25 may also comprise buffering agents. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

30 Dosage forms for topical or transdermal administration of an inventive pharmaceutical composition include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants or patches. The active component is admixed under

sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required. Ophthalmic formulation, ear drops, eye drops are also contemplated as being within the scope of this invention.

5 The ointments, pastes, creams and gels may contain, in addition to an active compound of this invention, excipients such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

10 Powders and sprays can contain, in addition to the compounds of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants such as chlorofluorohydrocarbons.

15 Transdermal patches have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms can be made by dissolving or dispensing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate can be controlled by either providing a rate controlling membrane or by dispersing the compound in a polymer matrix or gel.

20 Colloidal dispersion systems include liposomes, nanocapsules, microspheres, beads, lipid-based systems, oil/water emulsions, micelles, and mixed micelles. Liposomes can be used as delivery vehicles for RNA, DNA, viruses and viral particles (Fraley *et al. Trends Biochem. Sci.* 6:77, 1981; Mannino *et al. Biotechniques*, 6:682, 1988)

25 Kits including the components of the present invention are also provided by the invention. Kits may comprise components including nucleic acids, nucleic acids containing immunostimulatory sequences, antigenic molecules, nucleic acid binding molecules, antigenic molecules associated with nucleic acid-binding molecules, nucleic acids containing immunostimulatory sequences and binding sites for nucleic acid-binding molecules, pharmaceutically acceptable preparations of the preceding components and mixtures thereof.

30 For dosages of compositions of the present invention, those of ordinary skill in the arts will be familiar with or can determine without undue experimentation, proper dosages for administration of the compositions.

Encapsulation

In a preferred embodiment, inventive compositions comprising antigens and immunostimulatory nucleic acids are provided in association with an encapsulation device (see, for example, U.S.S.N. 60/169,330 entitled "Controlled Delivery of Antigens" filed Dec. 6, 1999, incorporated by reference herewith). Preferred encapsulation devices are biocompatible, are stable inside the body so that microorganisms are not released until after the encapsulation device reaches its intended destination (e.g. mucosal lining of the gut, endocytosis by antigen-presenting cells (APC)). For example, preferred systems of encapsulation are stable at physiological pH and degrade at acidic pH levels comparable to those found in the digestive tract or endosomes of APCs. Particularly preferred encapsulation compositions included but are not limited to ones containing liposomes, polylactide-co-glycolide (PLGA), chitosan, synthetic biodegradable polymers, environmentally responsive hydrogels, and gelatin PLGA nanoparticles. Inventive compositions may be encapsulated in combination with one or more adjuvants, targeting entities, or other agents including, for example, pharmaceutical carriers, diluents, excipients, oils, etc. Alternatively or additionally the encapsulation device itself may be associated with a targeting entity and/or an adjuvant.

In one particularly preferred embodiment of the invention, the encapsulation device comprises a live, preferably attenuated, infectious organism. (i.e., a microbe such as a bacterium or a virus) Inventive antigenic compositions may be introduced into the organism by any available means. In preferred embodiments of the invention, the organism is genetically engineered so that it synthesizes the antigen or antigen fragments itself. For example, genetic material encoding a protein antigen or peptide antigen fragment may be introduced into the organism according to standard techniques (e.g., transfection, transformation, electroporation, injection, etc.) so that it is expressed by the organism and the antigen or fragment produced. In particularly preferred embodiments of the invention, the protein or peptide antigen is engineered to be secreted from the organism (see, for example, WO98/23763. Those of ordinary skill in the art will appreciate that analogous systems can be engineered using any of a variety of other microbial or viral organisms. Any such system may be employed in the practice of the present invention.

The advantages of utilizing an organism as an encapsulation system include (i) integrity of the system prior to endocytosis, (ii) known mechanisms of endocytosis (often including targeting to particular cell types), (iii) ease of production of the delivered antigen or antigen fragments (typically made by the organism. experimental
5 accessibility of the organisms, including ease of genetic manipulation, ability to guarantee release (e.g., by secretion) of the antigen or antigen fragment after endocytosis, and the possibility that the encapsulating organism will also act as an adjuvant.

Oligonucleotides can alternatively be encapsulated in liposomes or virosomes
10 using well-known techniques. The present invention is further illustrated by the following Examples which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, published patent applications, and co-pending patent applications) cited throughout this application are hereby expressly incorporated by reference.

15

Methods of Administration

Formulations can be delivered to a patient by any available route including for example enteral, parenteral, topical (including nasal, pulmonary or other mucosal route), oral or local administration. Particularly preferred methods of administration
20 include oral, nasal, transdermal and subcutaneous routes. The compositions are preferably administered in an amount effective to elicit production of IgG while minimizing IgE mediated responses. For compositions of the present invention containing bacteria, administration is preferably delivered orally.

25 Uses

The compositions of the present invention may be employed to treat or prevent allergic reactions in any animal. Preferably, the animal is a domesticated mammal (e.g., a dog, a cat, a horse, a sheep, a pig, a goat, a cow, etc); more preferably, it is a human. Any individual who suffers from allergy, or who is
30 susceptible to allergy, may be treated. It will be appreciated that an individual can be considered susceptible to allergy without having suffered an allergic reaction to the particular antigen in question. For example, if the individual has suffered an allergic

reaction to a related antigen (e.g., one from the same source or one for which shared allergies are common), that individual will be considered susceptible to allergy to the relevant antigen. Similarly, if members of an individual's family are allergic to a particular antigen, the individual may be considered to be susceptible to allergy to that antigen. More preferably, any individual who is susceptible to anaphylactic shock upon exposure to food allergens, venom allergens or rubber allergens may be treated according to the present invention.

The compositions of the present invention may be formulated for delivery by any route. Preferably, the compositions are formulated for injection, ingestion, or inhalation.

Examples

Materials and Methods

MICE AND MATERIALS: Female C3H/HeJ mice, 3 weeks of age (immediately after weaning), were purchased from the Jackson Laboratory (Bar Harbor, Me) and maintained on regular mouse chow under specific pathogen-free conditions. Guidelines for the care and use of the animals were followed (Institute of Laboratory Animal Resources Commission on Life Sciences, *National Academy Press*, 1996).

Homogenized cow's milk (CM; GAF Seelig Inc) was used. Cholera toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, Calif). Concanavalin (Con A) and albumin, human-dinitrophenyl (DNP)-albumin were purchased from Sigma (St Louis, Mo). Antibodies for ELISAs were purchased from the Binding Site Inc or PharMingen (San Diego, Calif). Anti-DNP IgE was purchased from Accurate Scientific Inc.

SENSITIZATION AND CHALLENGE BY ORAL ADMINISTRATION OF ANTIGEN: Mice were sensitized intragastrically with CM plus CT as an adjuvant and boosted 5 times at weekly intervals. Intragastric feeding was performed by means of a stainless steel blunt feeding needle (Fine Science Tool Inc.) To determine the optimum sensitizing dose, mice received 0.01 mg (equivalent to the milk protein contained in homogenized CM) per gram of body weight (very low dose), 0.1 mg/g (low dose), 1.0 mg/g (medium dose), or 2 mg/g (high dose) of CM together with 0.3 µg/g of CT. The

CM/CT mixtures were administered in PBS at a final volume of 0.03 mL/g body weight. Control mice received CT alone or were left untreated. Six weeks after the first feeding, mice were fasted over night and challenged intragastrically with 2 doses of CM (30 mg/mouse) given 30 minutes apart.

5 MEASUREMENT OF CM-SPECIFIC IGE IN SERA: Blood was obtained weekly from the tail vein during the sensitization period and 1 day before challenge. Sera were collected and stored at -80 °C. Levels of CM-specific IgE were measured by ELISA as described previously (Li et al., *J Immunol.* 160:1378-84, 1998). Immulon II 96-
10 well plates (Dynatech Laboratories, Inc. Chantilly, Va) were coated with 20 µg-mL purified cow milk protein (CMP) (Ross Laboratories, Columbus, Ohio) in coating buffer, pH 9.6 (Sigma). After overnight incubation at 4 °C, plates were washed 3 times with PBS/0.05% Tween 20 and blocked with 1% BSA-PBS for 1 hour at 37°C. After washing 3 times, serum samples (1:10 dilutions) were added to the plates and incubated overnight at 4°C. Plates were then washed, and 100 µL of donkey anti-goat
15 IgG antibody conjugated with peroxidase (0.3 µg/mL) was added for an additional 1 hour at 37 °C. The reactions were developed with TMB (Bio-Rad Laboratories, Hercules, Calif) for 30 minutes at room temperature (RT), stopped with the addition of 1 N H₂SO₄, and read at 450 nm. The levels of IgE were calculated by comparison with a reference curve generated by using mouse mAbs (anti-DNP IgE), as previously
20 described (Li et al., *J Immunol.* 160:1378-84, 1998). All analyses were performed in duplicate.

 ASSESSMENT OF HYPERSENSITIVITY RESPONSES: Symptoms of systemic anaphylaxis appeared within 15 to 30 minutes and reached a peak at 40 to 50 minutes after the first symptoms appeared. Symptoms were evaluated by using a scoring
25 system modified slightly from previous reports and scored as follows: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3 = wheezing, labored respiration, and cyanosis around the mouth and the tail; 4 = no activity after prodding or tremor and convulsion; and 5 =
30 death.

 DETECTION OF VASCULAR LEAKAGE: Immediately before the second intragastric challenge with CM, 2 to 4 from each group received 100 µL of 0.5%

Evan's blue dye by tail vein injection. Footpads and intestines of mice were examined for signs of vascular leakage (visible blue color) 30 to 40 minutes after dye/antigen administration.

5 DETERMINATION OF PLASMA HISTAMINE LEVELS: Thirty minutes after challenge, blood was collected into chilled tubes containing 30 to 40 μ L of 7.5% potassium-EDTA. After centrifugation (1500 rpm) for 10 minutes at 4 °C, plasma aliquots were collected and frozen at - 80 °C. Histamine levels were determined by using an enzyme immunoassay kit (ImmunoTECH Inc), as described by the manufacturer.

10 PASSIVE CUTANEOUS ANAPHYLAXIS (PCA) TEST: Sera were obtained from 4 to 6 mice sensitized to CM (1 mg/g) plus CT and pooled. PCA tests were performed as previously described (Saloga et al., *J. Clin. Invest.* 91:133-40, 1993), with slight modification. Briefly, the abdomens of naïve mice were shaved 1 day before intradermal injection of 50 μ L of heated (56 °C for 3 hours) and unheated sera (1:5
15 dilution). Control mice received an equal amount of diluted naïve serum. Twenty four hours later, mice were injected intravenously with 100 μ L of 0.5% Evan's blue dye, immediately followed by an intradermal injection of 50 μ L of CMP (4 mg/mL). Thirty minutes after the dye/CMP injection, the mice were killed, the skin of they belly was inverted, and reactions were examined for visible blue color. A reaction
20 was scored as positive if the bluing of the skin at the injection sites was greater than 3 mm in diameter in any direction.

DETERMINATION OF SERUM ANTIGEN CONCENTRATION: To analyze intestinal permeability to casein, blood was collected from CM-sensitized (1 mg/g plus CT) or control mice 3 hours before and 30 to 40 minutes after intragastric challenge with
25 CM. Sera were prepared and stored at -80 °C. Levels of immunologically active casein in serum were measured by inhibition ELISA as previously described (Sampson et al., *J Pediatr* 118:520-5, 1991). Briefly, Immulon II 96-well plates were coated with 0.1 μ g/mL of casein in coating buffer (Sigma). After overnight incubation at 4 °C, plates were washed with 0.002 mol/L imidazole/0.02% Tween 20
30 and blocked with 0.07% ovalbumin at RT for 1 hour. Serum samples (1:20 dilution) or casein standards (8 dilutions from 30 μ g/mL to 0.1 μ g/mL) were incubated with rabbit anti-casein (1:150,000 dilution) antisera (Ross Laboratories) at 37 °C for 2

hours and were then added to the plates (100 mL/well). After incubation for 1 hour at RT, plates were washed. One hundred microliters of horseradish peroxidase-labeled goat anti-rabbit IgG (1:500 dilution; Sigma) was added and incubated for 1 hour at RT. The plates were subsequently washed, and TMB microwell peroxidase substrate (KPL, Gaithersburg, Md) was added and incubated for 15 minutes at RT. The reaction was stopped by the addition of TMB One Component Stop Solution (KPL) and read at 450 nm. The casein concentrations were determined by comparison with a standard curve.

HISTOLOGY: Mast cell degranulation during systemic anaphylaxis was assessed by examination of ear samples collected immediately after anaphylaxis-related death or 40 minutes after challenge from surviving mice as previously described (Snider et al., *J. Immunol* 153:647-57, 1994). Tissues were fixed in 4% phosphate-buffered formaldehyde (pH 7.2), and 5 µm paraffin sections were stained with toluidine blue or Giemsa stain. A degranulated mast cell was defined as a toluidine - or Giemsa-positive cell with 5 or more distinct stained granules completely outside of the cell. One section from each of 3 sites of each mouse ear was examined by light microscopy at 400X magnification by an observer unaware of their identities. Two hundred to 400 mast cells were classified for each ear sample. For assessment of intestinal mast cell degranulation, jejunal samples were fixed in Carnoy's solution and stained with toluidine blue or Giemsa.

For assessment of pathologic alterations, jejunum and lung samples were fixed in neutral-buffered formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H and E) and periodic acid-Schiff (PAS) reagent.

Mice were tested for immediate active cutaneous hypersensitivity (IACH) reactions by intradermal skin test 6 weeks after the initial sensitization with CM (1 mg/g plus CT), as previously described with a slight modification (Saloga et al., *J Clin Invest* 91:133-40, 1993; Hsu et al., *Clin Exp Allergy* 26:1329-37, 1996). Briefly under anesthesia the skin of the belly was shaved 1 day before the test. For each skin test, 50 µL of CMP (4 mg/mL) was injected intradermally with a 30-gauge needle while the skin was stretched taut. Antigen concentrations were determined by serial titration to produce consistent wheal reactions. PBS was used as a negative control.

The wheal reactions were assessed 30 minutes after intradermal injection with CM. A reaction was scored as positive if the wheal diameter was greater than 3 mm in any direction. Evaluations of wheal formation were carried out in a blinded fashion.

5 QUANTITATION OF CYTOKINE PROTEINS: Spleens were removed from mice allergic to CM after challenge. Cells were isolated and suspended in complete culture medium (RPMI-1640 plus 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine). Cell suspensions were cultured in 24-well plates (2 X 10⁶/well/mL) in the presence or absence of CMP (50 µg/mL) or Concanavalin A (Con A: 2 µg/mL). The supernatants were collected after 72 hours of culture. Levels of IFN-γ, IL-4, and 10 IL-5 were determined by ELISA, according to the manufacturer's instructions (Pharmigen) and as previously described (Li et al., *J Immunol* 157:3216-9, 1996; Li, et al., *J Immunol*. 160:1378-84; 1998).

15 STATISTICAL ANALYSIS: Statistical significance (P < 0.05) was determined by t test, ANOVA, or Mann-Whitney U test (rank-sum test). All statistical analyses were performed with GraphPad Prism (GraphPad Prism Software, Inc. San Diego, Calif).

Results

CM-SPECIFIC IgE RESPONSES AFTER INTRAGASTRIC CM SENSITIZATION: To investigate the kinetics of IgE production in the development of CMH, serum CM-specific IgE was monitored weekly by ELISA. Mice sensitized with the medium dose 20 (1 mg/g) of CM plus CT developed significant (P > 0.01) increases in antigen-specific IgE by 3 weeks, which peaked at 6 weeks after the initial sensitization. Significantly lower levels of antigen-specific IgE were induced by both a higher dose (2 mg/g) and lower doses (0.01, 0.1 mg) of CM plus CT.

25 CHARACTERIZATION OF SYSTEMIC ANAPHYLAXIS AFTER CHALLENGE: Six weeks after initial sensitization (the time of peak IgE response), the mice were challenged intragastrically with CM. Systemic anaphylactic symptoms were evident within 15 to 30 minutes. The severity of anaphylaxis was scored as indicated above. Consistent with the IgE responses, the most severe reactions were also observed in 30 mice sensitized with the medium dose (1 mg/g) of CM plus CT. Mice sensitized with the higher and lower doses showed weaker reactions, indicating that the severity of anaphylaxis in this model was associated with the concentration of CM-specific IgE.

CT sham-sensitized mice and naïve mice showed no anaphylactic reactions after CM challenge. These findings demonstrate that the antigen dose influences the intensity of response to oral sensitization and challenge. Taken together, we concluded that sensitization with CM at the dose of 1 mg/g body weight was optimal, and this dose was used in the remainder of the studies.

VASCULAR LEAKAGE AFTER CHALLENGE OF SENSITIZED MICE: Increased vascular permeability, induced by vasoactive mediators such as histamine, is a hallmark of systemic anaphylaxis. Extensive Evan's blue dye extravasation was evident in footpads of CM-sensitized mice, but not CT sham-sensitized mice, after oral challenge (data not shown).

ELEVATED PLASMA HISTAMINE LEVEL AFTER CHALLENGE OF SENSITIZED MICE: Plasma histamine levels were significantly increased in CM-sensitized (1 mg/g plus CT) mice (4144 ± 1244 nmol/L) after challenge when compared with CT sham-sensitized (661 ± 72 nmol/L) and naïve mice (525 ± 84 nmol/L). These results suggest that histamine is one of the major mediators involved in the anaphylaxis in this model.

INCREASED MAST CELL DEGRANULATION AFTER CHALLENGE OF SENSITIZED MICE: Histologic analysis of mouse ear tissue showed many degranulated mast cells in CM-sensitized and challenged mice, but not control mice (data not shown). The percentage of degranulated mast cells was approximately 9 times greater than that in the PCT sham-sensitized group. These results were consistent with the findings of elevated levels of plasma histamine after challenge of CM-sensitized mice, demonstrating that mast cell degranulation and consequent histamine release are involved in the induction of systemic anaphylaxis in this model.

PCA REACTIONS: Because antigen-specific IgE levels were associated with the severity of anaphylaxis, we hypothesized that IgE, and not IgG1, was responsible for the induction of CMH. To confirm this possibility, PCA testing was performed. Injection PCA reactions, which were eliminated by heat inactivation of immune sera. These results demonstrate that IgE is the reaginic antibody in this model.

Table 2				
DONOR IMMUNIZATION	HEAT INACTIVATION	DIAMETER (MM) MEAN \pm SEM	POSITIVE REACTION N/TOTAL	%
CM+CT	-----	8.87 \pm 1.14*	8/8	100
CM \pm CT	+	0.58 \pm 0.42	0/6	0
Naive	-----	0.7 \pm 0.37	0/5	0

CHARACTERIZATION OF INTESTINAL REACTIONS: Increased intestinal permeability after intragastric CM challenge. Altered permeability was assessed in 2 ways: increased mucosal permeability by measurement of serum casein levels and increased intestinal vascular permeability by Evan's blue dye extravasation. Before intragastric challenge with CM, serum casein levels were comparable in CM-sensitized mice (41 \pm 20 ng/mL) and in CT control mice (42 \pm 12 ng/mL). However, 30 to 40 minutes after challenge, levels of serum casein in CM-sensitized mice (7890 \pm 256 ng/mL) undergoing anaphylaxis were significantly higher than those of the control mice (205 \pm 23 ng/mL), demonstrating that increased mucosal permeability is a characteristic of this model. Intestines from CM-sensitized mice challenged intragastrically and injected with Evan's blue exhibited dark blue discoloration, whereas naive mice receiving the same antigen/dye administration did not. These results indicate that mucosal and vascular permeability are increased in intestines in this model of milk allergy.

HISTOLOGIC ANALYSIS OF INTESTINE: Histologic examination of the small intestines revealed marked vascular congestion and edema of the lamina propria and, in some areas, sloughing of enterocytes at the tips of the villi (data not shown). The histologic appearance was essentially the same as that described in intestinal anaphylaxis in rats (D'Inca et al., *Int Arch Allergy Appl Immunol* 91:270-7, 1990; Levine et al., *Int Arch Allergy Immunol* 115:312-5, 1998). Only a small number of mast cells were observed in the intestines of normal and allergic mice, and most of these were scattered within the serosa. Mast cells were not present within villi and were rarely observed at the base of the crypts. This finding is consistent with prior

histochemical and immunohistochemical studies of normal mouse intestines (Carroll et al., *Int Arch Allergy Appl Immunol* 74:311-7, 1984; Scudamore et al., *Am J Pathol* 150:1661-72, 1997). In contrast to the significant numbers of mast cells detected in skin of the same animals, the small numbers of intestinal mast cells precluded analysis of anaphylaxis-induced degranulation.

CHARACTERIZATION OF PULMONARY RESPONSES: We observed that CM-induced immediate reactions in this model were frequently accompanied by respiratory symptoms, such as wheezing and labored respiration. Histologic examination revealed that lungs from these animals were markedly inflamed and contained large numbers of perivascular and peribronchial lymphocytes, monocytes, and eosinophils when compared with control mice (data not shown). Increased numbers of PAS-positive goblet cells were present in bronchi and bronchioles. In some instances the bronchial lumen appeared to be filled with mucus. These lungs exhibited essentially the same appearance as lungs from mice sensitized intraperitoneally and challenged by the intratracheal route (Li et al., *J Immunol*, 160:1378-84, 1998, Gavett et al., *Am J Physiol* 272:L253-61, 1997).

INDUCTION OF IACH AFTER ORAL CM CHALLENGE IN SENSITIZED MICE: It has been demonstrated that IACH reactions are associated with IgE-induced mast cell degranulation. Thus the IACH has been used for the rapid evaluation of immediate allergic reactions (Saloga et al., *J Clin Invest* 91:133-40, 1993; Hamelmann et al., *J Exp Med*, 183:1719-29, 1996). Because the first sign of reactions after intragastric challenge was scratching in most of the mice, we performed skin tests at the time of challenge to characterize the skin reactions. Five of 7 (71.4%) CM-sensitized mice experienced IACH-positive reactions after intradermal CMP injection. In contrast, IACH reactions were not induced in CM-sensitized mice after intradermal injection of PBS or in naïve mice after intradermal injection of CMP.

INCREASED TH2 - TYPE CYTOKINE RESPONSES: To determine the role of T cells and cytokines in the development of CMA, we examined the production of cytokines by spleen cells from mice allergic to CM stimulated in vitro with CMP. After 72 hours in culture, IL-4 and IL-5 levels were significantly ($P < 0.001$) increased in CMP-stimulated cultures (44 and 68 pg/mL, respectively) when compared with unstimulated cells (undetectable). In contrast, IFN- γ levels in CM-stimulated and

unstimulated spleen cells (10 and 14 pg/mL, respectively) were essentially the same (P > 0.5).

Example 1

5 A Murine Model of Peanut Anaphylaxis

Introduction

This Example describes the development of a mouse model system for anaphylactic peanut (PN) allergy. This system may be employed in accordance with the present invention to identify and characterize compositions containing peanut
10 antigen fragments, such as those described in the following Examples, capable of desensitizing and/or vaccinating individuals from peanut allergy.

Materials and Methods

MICE AND REAGENTS: Female C3H/HeJ mice, 5-6 weeks of age were purchased
15 from the Jackson Laboratory (Bar Harbor, ME) and maintained on PN-free chow, under specific pathogen-free conditions. Standard guidelines, Institute of Laboratory Animal Resources Commission of Life Sciences NRC: *National Academy Press*, 1996, for the care and use of animals were followed

Freshly ground whole PN was employed as antigen (Ag). Crude PN extract. Ara
20 h 1 and Ara h 2 were prepared as described previously (Burks, et al., *Adv. Exp. Med. Biol.*, 289:295-307, 1991; Burks, et al., *J Allergy Clin. Immunol.*, 90:962-969, 1992). Cholera Toxin (CT) was purchased from List Biological Laboratories, Inc (Campbell, CA). Concanavalin A (Con A), and albumin, and human-dinitrophenyl (DNP-albumin) were purchased from Sigma (St. Louis, MO). Antibodies for ELISAs were
25 purchased from the Binding Site Inc. or Pharmingen (San Diego, CA). Anti-DNP IgE was purchased from Accurate Scientific Inc. (New York).

INTRAGASTRIC SENSITIZATION AND CHALLENGE: Mice were sensitized by intragastric (ig) feeding with freshly ground whole PN on day 0 and boosted on day 7. Intragastric feeding was performed by means of a stainless steel blunt feeding needle
30 as described previously (Li et al., *J. Immunol.*, 153:647-657, 1994). To determine an optimum sensitization dose, mice received 5 mg/mouse (low dose), or 25 mg/mouse (high dose) of PN together with 10 µg/mouse of CT. Three weeks following the

initial sensitization. mice were challenged ig with crude PN extract 10 mg/mouse in 2 doses at 30-40 min. intervals. Sham sensitized mice were challenged in the same manner. Mice surviving the first challenge were re-challenged at weeks 5.

5 Additional mice were sensitized ig with Ara h 2, one of the major PN allergens. 1 mg/mouse, together with CT, and boosted 7 and 21 days later.

ASSESSMENT OF HYPERSENSITIVITY REACTIONS: Anaphylactic symptoms were evaluated 30-40 minutes following the second challenge dose utilizing a scoring system, modified slightly from previous reports (Li et al., *J. Allergy Clin. Immunol.* 103:206-214; Poulsen et al., *Clin. Allergy*; 17:449-458, 1987; McCaskill et al.,
10 *Immunology*, 51:669-677, 1984): 0 - no symptoms: 1-scratching and rubbing around the nose and head; 2 - puffiness around the eyes and mouth, diarrhea, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate: 3 - wheezing, labored respiration, cyanosis around the mouth and the tail: 4 - no activity after prodding, or tremor and convulsion; 5 - death.

15 MEASUREMENT OF PLASMA HISTAMINE LEVELS: To determine plasma histamine levels, blood was collected 30 minutes after the second ig challenge. Plasma was prepared as previously described (Li et al., *J. Immunol.* 162:3045-3052, 1999; Li et al., *J. Allergy Clin. Immunol.* 103:206-214, 1999) and stored at -80 °C until analyzed. Histamine levels were determined using an enzyme immunoassay kit (ImmunoTECH
20 Inc., ME), as described by the manufacturer.

MEASUREMENT OF PN-SPECIFIC IGE, IgG1 AND IgG2 : Tail vein blood was obtained at weekly intervals following initial sensitization. Sera were collected and stored at - 80 °C. Levels of PN-specific IgE were measured by ELISA as described previously (Li et al., *J. Immunol.* 160:1378-1384, 1998), with slight modification.
25 Briefly, ImmulonII 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 20 µg/ml crude PN extract in coating buffer, pH 9.6 (Sigma, St. Louis, MO). After overnight incubation at 4 °C, plates were washed and blocked with 1% BSA-PBS for 1 hour at 37°C. After 3 x washings, serum samples (1:10 dilutions) were added to the plates and incubated overnight at 4 °C. Plates were then washed 3 x
30 and 100 µl of goat anti-mouse IgE (0.3 µg/ml) was added to each well. The plates were incubated for 2 hrs at 37°C. After 3 x washings, 100 µl of biotinylated donkey anti-goat IgG (0.3 µg/ml) was added to each well for an additional 1 hr incubation at

room temperature (RT). After 5 washings, 100 µl of avidin peroxidase (Sigma, St. Louis, MO, CA) (1:1000 dilution) was added for an additional 30 min. at RT. After 8 washings, the reaction was developed with ABTS (KPL, Gaithersburg, MD) for 30 min. at RT and read at 405 nm. Levels of IgE were calculated by comparison with a reference curve generated by using mouse monoclonal antibodies, anti-DNP IgE (Accurate Scientific Inc. NY, USA) as described previously (Li et al., *J. Immunol.* 162:3045-3052, 1999; Li, et al., *J. Immunol.* 103:206-214, 1999).

For measurement of PN-specific IgG1 and IgG2a, plates were coated with crude PN extract and then blocked and washed in the same manner as above. Samples (1:50 dilution) were added to the plates and incubated overnight at 4 °C. Plates were then washed and biotinylated rat anti-mouse IgG1 or IgG2a monoclonal antibodies (1 µg/ml; PharMingen San Diego, CA) were added to the plates for detection of IgG1 and IgG2a respectively. Plates were incubated for an additional 1 hr at room temperature. After washings, avidin peroxidase was added for an additional 15 min. at RT. After 8 washings, the reactions were developed with ABTS (KPL) for 30 min. at RT and read at 405 nm.

To further characterize specific IgE responses to the major PN allergens, plates were coated with purified Ara h 1 or Ara h 2. The remaining steps were performed as described above.

PASSIVE CUTANEOUS ANAPHYLAXIS (PCA) TESTING: Sera were obtained from 4 to 6 mice sensitized with low dose of PN and pooled. PCA tests were performed as previously described with slight modification. Briefly, the abdomens of naive mice were shaved one day prior to intradermal (id) injection of 50 µl heated (56 °C for 3 hr) or unheated sera (1:5 dilution). Control mice received an equal amount of diluted naive serum. Twenty-four hours later, mice were injected intravenously with 100 µl of 0.5% Evan's blue dye, immediately followed by an id injection of 50 µl of crude PN extract (4 mg/ml). Thirty-min. following the dye/antigen (Ag) administration, the mice were sacrificed, the skin of the belly was inverted, and reactions were examined for visible blue color. A reaction was scored as positive if the bluing of the skin at the injection sites was > 3 mm in diameter in any direction.

HISTOLOGY: Mast cell degranulation during systemic anaphylaxis was assessed by examination of ear samples collected immediately after anaphylactic death or 40

min. after challenge from surviving mice as previously described (Li et al., *J. Immunol.* 162:3045-3052, 1999; Snider et al., *J. Immunol.*, 153:647-657, 1994). Tissues were fixed in 10% neutral buffered formalin and 5- μ m toluidine blue or Giemsa stained paraffin sections from three sites of each mouse ear was examined by light microscopy at 400 X by an observer unaware of their identities. A degranulated mast cell was defined as a toluidine blue or Giemsa-positive cell with five or more distinct stained granules completely outside of the cell. Four hundred mast cells in each ear sample were classified.

PROLIFERATION ASSAYS: Spleens were removed from PN sensitized and naive mice after re-challenge at week 5. Spleen cells were isolated and suspended in complete culture medium (RPMI 1640 plus 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% glutamine). Spleen cells (1×10^6 well in 0.2 ml Bock, et al., *J. Allergy Clin. Immunol.* 62:327-334, 1978) were incubated in triplicate cultures in microwell plates in the presence or absence of crude PN extract. Ara h 1 or Ara h 2 (10, 50 μ g/ml). Cells stimulated with Con A (2 μ g/ml) were used as a positive control. Four days later, the cultures received an 18-hr pulse of 1 μ Ci per well of Yunginger et al. (*JAMA* 260:1450-1452, 1988). H-thymidine. The cells were harvested and the incorporated radioactivity was counted in a β scintillation counter. The results were expressed as counts per minute (cpm).

TWO-DIMENSIONAL GEL ELECTROPHORESIS AND IMMUNOBLOTTING: Two-dimensional gel electrophoresis was employed to separate PN proteins using previously described methods with slight modifications (Burks et al., *J. Allergy Clin. Immunol.* 90:962-969, 1992; O'Farrell et al., *Cell*. 12:1133-1141, 1977; Hochstrasser et al., *Anal. Biochem.*, 173:424-435, 1988). The first dimension consisted of an isoelectric focusing gel in glass tubing. After making the gel mixture with a pH gradient of 3.5-10 (Bio Rad Laboratories) 200 μ g samples were loaded and focused with a BioRad Protean II xi 2-D cell at 200 V for 2 hours, 500 V for 2 hours and 800 V overnight. The second dimension gel, sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), employed an 18% polyacrylamide separating and a 4% stacking gel as previously described (Burks et al., *J. Allergy Clin. Immunol.*, 90:962-969, 1992; Laemmli et al., *Nature*, 227:680-685, 1970). Electrophoresis was performed for 18

hours at 25 mA per 14 cm by 12 cm gel with a set limit of 150 V in a Hoefer Apparatus (Pharmacia Biotech).

Proteins were transferred from the separating gel to a 0.22 μ m nitrocellulose membrane in a Tris-Glycine buffer containing 20% methanol. The procedure was performed in a Hoefer transfer unit for 14 hours at 100 mA. To assure proper protein separation and quality of transfer, one nitrocellulose membrane from each pair was stained with Amido-Black, while both polyacrylamide gels were stained with Coomassie Brilliant Blue.

After removal from the transblot apparatus, the nitrocellulose membranes were placed in blocking solution (PBS containing 0.5% gelatin, 0.05% Tween and 0.001% thimerosal) overnight at RT on a rocking platform. The nitrocellulose blot was then washed three times with PBS containing 0.05% Tween (PBST) and incubated with pooled sera from highly sensitive PN-allergic patients [1:10 dilution in a blocking solution] for two hours at RT. After rinsing and washing four times with PBST, alkaline phosphatase-conjugated goat anti-human IgE (KPL, 0.5 μ g/ml) was added and incubated at RT for 2 hours. After rinsing and washing with PBST four times, the blot was developed with BCIP/NBT Phosphatase Substrate System (KPL) for 5 min. The reaction was stopped by washing the nitrocellulose membrane with distilled water and the blot was air-dried.

For characterization of mouse IgE antibody binding to allergenic PN proteins, the nitrocellulose blot prepared as above. The blot was incubated with pooled sera from PN-sensitive mice [1:10 dilution] overnight at RT, followed by extensive washes with PBST and another overnight incubation in 0.75 μ g/ml sheep anti-mouse IgE (The Binding Site, UK). The blot was then washed 4 times and 0.3 μ g/ml horseradish peroxidase conjugated donkey anti-sheep IgG (The Binding Site, UK) was added. After 2 hours incubation at RT, the blot was washed and developed with TMB Membrane Substrate Three Component System (KPL) for 15 min., washed with distilled water, and air-dried.

Results

SYSTEMIC ANAPHYLACTIC REACTIONS FOLLOWING INTRAGASTRIC CHALLENGE:
Three weeks following the initial sensitization, mice were twice challenged ig with

crude PN extract at 30-40 intervals. Systemic anaphylactic symptoms were evident within 10-15 min following the first challenge, and the severity of the anaphylaxis was evaluated at 30-40 min. after the second challenge. The initial reactions were expressed as cutaneous reactions, puffiness around the eyes and mouth, and/or diarrhea followed by respiratory reactions such as wheezing and labored respiration. The most severe reactions were loss of consciousness and death. We further observed that mice sensitized with the low dose (5 mg/mouse + CT) of whole PN exhibited more severe reactions than those sensitized with the high dose (25 mg/mouse + CT). Fatal or near fatal anaphylactic shock occurred in 12.5% of low dose sensitized mice but in none of the high dose sensitized mice. Sham sensitized and naïve mice did not show any symptoms of anaphylaxis.

Two weeks following the first challenge, the surviving mice were re-challenged. Systemic anaphylactic reactions were again provoked, and were more severe than those induced by the first challenge. The low sensitization dose group also exhibited the most severe reactions at this time point, with a 21% mortality rate. These results showed that the initial sensitizing dose determined the intensity of the hypersensitivity reactions. We concluded that sensitization with PN at the dose 5 mg/mouse (low dose) was optimal and this dose was used for subsequent studies.

INCREASED MAST CELL DEGRANULATION AND HISTAMINE RELEASE FOLLOWING IG-CHALLENGE: The percentage of degranulated mast cells in ear tissues were significantly greater in PN sensitized mice than in controls following PN-challenge. Consistent with this finding, plasma histamine levels were also significantly increased in PN sensitized mice compared with CT sham sensitized and naive mice. These results suggest that histamine, and probably other mediators released from mast cells contributed to the symptoms of PN-induced anaphylaxis.

KINETICS AND ISOTYPE PROFILE OF PN-SPECIFIC ANTIBODIES FOLLOWING PN-SENSITIZATION AND CHALLENGE: To explore the humoral immune responses underlying the development of PN-induced hypersensitivity, sera from the different groups of mice were obtained weekly after ig sensitization and challenge. Levels of PN-specific antibody isotypes were determined by ELISA. IgE levels were significantly increased at week 1 through week 5 in mice sensitized with low dose PN (5 mg/mouse), and from week 2 through week 5 in mice sensitized high dose PN (25

mg/mouse). Furthermore, specific IgE levels in the low dose group were significantly higher than in the high dose at both week 3 (initial challenge) and week 5 (re-challenge), suggesting an association between IgE levels and severity of anaphylactic reactions.

5 In addition, PN-specific IgG1 levels were not significantly different between the high and low dose groups at weeks 3 and 5 (data not shown), suggesting that IgG1 was not associated with PN-hypersensitivity reactions in this model. In contrast to PN-specific IgE responses, IgG2a levels in the high dose group were significantly higher than in the low dose group at both weeks 3 and 5 (data not show) suggesting
10 that IgG2a was inversely related with the induction of PN-hypersensitivity.

PCA REACTIONS: Since Ag-specific IgE levels appeared positively correlated with the severity of anaphylaxis, we hypothesized that IgE was responsible for the induction of PN hypersensitivity in this model. To confirm this possibility, and to rule out IgG1-mediated anaphylaxis, which is known to occur in mice, PCA testing
15 was performed. Injection of immune sera from PN allergic mice, but not heat-inactivated immune sera, induced PCA reactions (Table 1). Elimination of PCA reactions by heat inactivation of immune sera demonstrated that IgE is the reagenic antibody in this model.

20

Table 1				
donor immunization	heat inactivation	diameter (mm)(mean±SE)	positive reactions n/total	%
PN + CT	----	8.28±1.20*	7/7	100
PN + CT	+	1.16±0.40	0/6	0
naïve	----	1.20±0.37	0/5	0

25

T-CELL PROLIFERATIVE RESPONSES TO WHOLE PN AND THE MAJOR PN ALLERGENS ARA H 1 AND ARA H 2 RESEMBLE THOSE OF HUMAN PNA: To characterize T cell responses to whole PN, or major PN allergens in this model, spleen cells from PN-allergic mice or naïve mice were cultured with crude PN extract, Ara h 1, or Ara h
30 2. Although cells from both PN-allergic mice and naïve mice showed significant

proliferative responses to Con A stimulation, cells from PN allergic mice, but not from naïve mice, exhibited significant proliferative responses to PN, Ara h 1, and Ara h 2 stimulation. These results demonstrated that the T cells responses to PN and the major PN allergens were similar to those observed in PN allergic patients (Shin et al.,

5 *J. Biol. Chem.* 273:13753-13759, 1998).

B-CELL IgE RESPONSES TO THE MAJOR PN ALLERGENS PN ALLERGENS ARA H 1 AND ARA H 2 RESEMBLE THOSE OF HUMAN PEANUT ALLERGY (PNA): To determine whether IgE from PN-allergic mice recognized the same major PN allergens as IgE from PN allergic patients, we measured IgE Ab against Ara h 1 and Ara h 2 in pooled sera of PN-allergic and naive mice. Both Ara h 1- specific and Ara h 2-specific IgE
10 were present in the sera of PN-allergic mice.

In addition, C3H/HeJ mice were also sensitized ig with the major PN allergen, Ara h 2 (1 mg/mouse + CT). Levels of Ara h 2 specific IgE were markedly increased at week 3 (298 ng/ml) peaked at week 4 (511 ng/ml) and remained elevated for a least
15 7 weeks (383 ng/ml). These results demonstrate that B cell IgE responses to PN allergens in this model resemble B cell IgE responses in human PNA both in vitro and in vivo.

COMPARISON OF PN ALLERGIC MOUSE AND PN ALLERGIC HUMAN IgE ANTIBODY BINDING TO THE MAJOR PN ALLERGEN ARA H 2: Following the detection of anti-Ara h
20 1 and anti-Ara h 2-specific IgE antibodies in pooled sera of PN-allergic mice, we next compared PN-allergic mouse and human IgE antibody binding to the major PN allergen Ara h 2 fractions by employing two-dimensional gel electrophoresis and immunoblotting. Human IgE recognizes 8 Ara h 2 isoforms which have been previously characterized (see Example 3). IgE from PN-sensitized mice recognized
25 the same Ara h 2 isoforms as human IgE. Furthermore, mouse IgE bound no additional Ara h 2 fractions. In addition, mouse IgE also recognized 2 of the 6 minor Ara h 3 isoforms recognized by human IgE.

Example 2

Mapping IgE Binding Sites in Peanut Antigens

Introduction

5 This Example describes the definition and analysis of IgE binding sites within peanut protein antigens. The information presented may be utilized in accordance with the present invention, for example, to prepare one or more antigen fragments, or collections thereof, lacking one or more peanut antigen IgE binding site. In general, any of a variety of methods (e.g., immunoprecipitation, immunoblotting, cross-linking, etc.) can be used to map IgE binding sites in antigens (see, for example, 10 methods described in Coligan et al. (eds) *Current Protocols in Immunology*, Wiley & Sons, and references cited therein, incorporated herein by reference). Generally, an antigen or antigen fragment (prepared by any available means such as, for example, chemical synthesis, chemical or enzymatic cleavage, etc.) is contacted with serum from one or more individuals known to have mounted an immune response against 15 the antigen. Where the goal is to map all observed IgE binding sites, it is desirable to contact the antigen or antigen fragment, simultaneously or serially, with sera from several different individuals since different epitopes may be recognized in different individuals. Also, different organisms may react differently to the same antigen or antigen fragments; in certain circumstances it may be desirable to map the different 20 IgE binding sites observed in different organisms.

It will be appreciated that an IgE binding site that is strongly recognized in the context of an intact antigen may not be strongly bound in an antigen fragment even though that fragment includes the region of the antigen corresponding to the binding site. As will be clear from context, in some circumstances an antigen fragment is 25 considered to contain an IgE binding site whenever it includes the region corresponding to an IgE binding site in the intact antigen; in other circumstances, an antigen fragment is only considered to have such a binding site if physical interaction has actually been demonstrated as described herein.

30 *Materials and Methods*

IGE IMMUNOBLOT ANALYSIS: Membranes to be blotted were prepared either by SDS-PAGE (performed by the method of Laemmli *Nature* 227:680-685, 1970) of

digested peanut antigen or by synthesis of antigen peptides on a derivativized cellulose membrane). SDS-PAGE gels were composed of 10% acrylamide resolving gel and 4% acrylamide stacking gel. Electrophoretic transfer and immunoblotting on nitrocellulose paper was performed by the procedures of Towbin (*Proc. Natl. Acad. Sci. USA* 76:4350-4354, 1979).

For mapping of human IgE binding sites, the blots were incubated with antibodies (serum IgE) from 15-18 patients with documented peanut hypersensitivity. Each of the individuals had a positive immediate skin prick test to peanut and either a positive, double-blind, placebo-controlled food challenge or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension). At least 5 ml of venous blood was drawn from each patient and allowed to clot, and the serum was collected. All studies were approved by the Human Use Advisory Committee at the University of Arkansas for Medical Sciences. Serum was diluted in a solution containing TBS and 1% bovine serum albumin for at least 12 H at 4 °C or for 2 h at room temperature. The primary antibody was detected with ¹²⁵I-labeled anti-IgE antibody (Sanofi Diagnostics Pasteur Inc., Paris, France).

For mapping of murine IgE binding sites, a blot containing overlapping 13mer peptides, offset by 2 amino acids, was incubated with serum from mice described in Example 2.

PEPTIDE SYNTHESIS: Individual peptides were synthesized on a derivativized cellulose membrane using Fmoc amino acid active esters according to the manufacturer's instructions (Genosys Biotechnologies, Woodlands, TX). Fmoc-amino acid derivatives were dissolved in 1-methyl-2-pyrrolidone and loaded on marked spots on the membrane. Coupling reactions were followed by acetylation with a solution of 4% (v/v) acetic anhydride in *N,N*-dimethyl formamide (DMF). After acetylation, Fmoc groups were removed by incubation of the membrane in 20% (v/v) piperidine in DMF. The membrane was then stained with bromophenol blue to identify the location of the free amino groups. Cycles of coupling, blocking, and deprotection were repeated until the peptides of the desired length were synthesized. After addition of the last amino acid in the peptide, the amino acid side chains were deprotected using a solution of dichloromethane/trifluoroacetic acid/triisobutylsilane

(1/10/0.05). Membranes were either probed immediately or stored at -20 °C until needed.

Results

Human IgE binding sites have previously been mapped for Ara h 1 (Burks et al., *J. Clin. Invest.* 96:1715-1721, 1995; USSN 90/141.220, filed August 27, 1998, each of which is incorporated herein by reference) and Ara h 2 (Stanley et al., *Arch. Biochem. Biophys.* 342:244-253, 1997; USSN 90/141.220, filed August 27, 1998, each of which is incorporated herein by reference). We have also mapped such epitopes for Ara h 3 (Rabjohn et al., *J. Clin. Invest.* 103:535-542, 1999; USSN 90/141.220, filed August 27, 1998, each of which is incorporated herein by reference). We have also mapped murine IgE binding sites for Ara h 2, by probing filters containing overlapping 20mers, offset by 5 amino acids, that span the Ara h 2 sequence with serum from mice sensitized to recombinant Ara h 2.

The results of these studies are summarized below in Tables (essential residues are underlined).

Table 2		
IgE Binding Epitopes in Ara h 1		
EPITOPE NUMBER	SEQUENCE	POSITION
1	AKSSPYQKKT	25-34
2	QEPDDLKOKA	48-57
3	LEYDPRLVYD	65-74
4	GERTRGROPG	89-98
5	PGDYDDDRRQ	97-106
6	PRREEGGRWG	107-116
7	REREEDWRQP	123-132
8	EDWRRPSHOQ	134-143
9	QPRKIRPEGR	143-152
10	TPGQFEDFFP	294-303
11	SYLOEFSRNT	311-320

5

10

12	<u>FNAEFNEIRR</u>	325-334
13	<u>EQEERGQRRW</u>	344-353
14	<u>DITNPINLRE</u>	393-402
15	<u>NNFGKLFVK</u>	409-418
16	<u>GTGNLELVAV</u>	461-470
17	<u>RRYTARLKEG</u>	498-507
18	<u>ELHLLGFGIN</u>	525-534
19	<u>HRIFLAGDKD</u>	539-548
20	<u>IDQIEKQAKD</u>	551-560
21	<u>KDLAFPGSGE</u>	559-568
22	<u>KESHFVSARP</u>	578-587
23	<u>PEKESPEKED</u>	597-606

15

20

Table 3 IgE Binding Epitopes in Ara h 2		
SEQUENCE OF HUMAN EPI TOPE (NUMBER)	SEQUENCE OF MOUSE EPI TOPE (NUMBER)	POSITION
<u>HASARQQWEL</u> (1)	LFLLA AH (1)	H15-24 M9-15
<u>QWELQGDRRC</u> (2)	RQQWELQGDRR (2)	H21-28 M19-29
<u>DRRCOSQLER</u> (3)	RCQSQLERA (3)	H27-36 M29-37
<u>LRPCEQHLMQ</u> (4)		H39-48
<u>KIQRDEDSYE</u> (5)	DEDSYERDP (4)	H49-56 M53-61
<u>YERDPYSPSQ</u> (6)	YERDPYSPS (5)	H59-64 M57-65
<u>SQDPYSPSPY</u> (7)	YSPSPYD (6)	H65-72 M69-75

5	<u>DRLOGROQEQ</u> (8)	QEQQQFK (7)	H117-122 M121-127
	<u>KRELRNLPQQ</u> (9)	KRELRNLPQ (8)	H127-132 MM127-135
		RNLPQQCGL (9)	M131-139
		CGLRAPQ (10)	M137-143
	<u>QRCDLDVESG</u> (10)	QRCDLDV (11)	H143-152 M143-149

Human Ara h 2 epitopes (6) and (7), and mouse Ara h 2 epitopes (5) and (6) were considered to be immunodominant because, in each case, the two epitopes combined contributed about 40-50% of the observed IgE reactivity (as determined by densitometric analysis of the blot). Human epitope (3) was also considered to be immunodominant, as it contributed as much as about 15% of the IgE reactivity. No other mouse or human epitope contributed more than about 10% of the reactivity.

15	Table 4		
	IgE Binding Epitopes in Ara h 3		
20	EPITOPE NUMBER (FRACTION OF PATIENTS WITH IGE THAT BIND)	SEQUENCE	POSITION
	1 (25%)	<u>IETWNPNNQEFECAG</u>	33-47
	2 (38%)	<u>GNIFSGFTPEFLEQA</u>	240-254
	3 (100%)	<u>VTVRGGLRILSPDRK</u>	279-293
	4 (38%)	DEDEYEYDEEDRRRG	303-317

Epitope 3 of Ara h 3 was designated as immunodominant because it was recognized by IgE in sera from all 10 patients tested.

Example 3
Collections of Ara h 2 Peptides

5/20 Native

A collection of 28 peptides, each 20 amino acids long and staggered by 5 amino acids, spanning the sequence of the native Ara h 2 protein (SEQ ID NO:2) was prepared as described above. Table 5 presents the sequences of the individual peptides:

Table 5		
5/20 Native Ara h 2 Peptides		
PEPTIDE NO	SEQ ID NO:	SEQUENCE
1		LTILVALALFLLAHASARQ
2		ALALFLLAHASARQQWELQ
3		LLAAHASARQQWELQGDRRC
4		ASARQQWELQGDRRCQSQLE
5		QWELQGDRRCQSQLERANLR
6		GDRRCQSQLERANLRPCEQH
7		QSQLERANLRPCEQHLMQKI
8		RANLRPCEQHLMQKIQRDED
9		PCEQHLMQKIQRDEDSYERD
10		LMQKIQRDEDSYERDPYSPS
11		QRDEDSYERDPYSPSQDPYS
12		SYERDPYSPSQDPYSPSPYD
13		PYSPSQDPYSPSPYDRRGAG
14		QDPYSPSPYDRRGAGSSQHQ
15		PSPYDRRGAGSSQHQRCCN
16		RRGAGSSQHQRCCNELNEF
17		SSQHQRCCNELNEFENNQR
18		ERCCNELNEFENNQRCEA

19		ELNEFENNQRCMCEALQQIM
20		ENNQRCMCEALQQIMENQSD
21		CMCEALQQIMENQSDRLQGR
22		LQQIMENQSDRLQGRQQEQQ
23		ENQSDRLQGRQQEQQFKREL
24		RLQGRQQEQQFKRELRNLPQ
25		QQEQQFKRELRNLPQQCGLR
26		FKRELRNLPQQCGLRAPQRC
27		RNLPQQCGLRAPQRCDLDVE
28		QCGLRAPQRCDLDVESGGRD

Each of these peptides was tested for its ability to stimulate T cells. Each peptide was tested, using standard different techniques, on 19 different T cell preparations. Positive scores, defined as a T cell stimulation index of > 2, are indicated by shading. As can be seen, peptides 1-9 (especially 3 and 4) and 18029 (especially 18-22 and 25-28) have significant T cell stimulation capability; peptides 10-17 do not show such activity.

5/15 Modified

A collection of 24 peptides, each (except for the last) 15 amino acids long and staggered by 5 amino acids, spanning the sequence of a modified Ara h 2 protein (SEQ ID NO: __), in which all IgE binding sites were disrupted by alanine substitution can be synthesized. Table 6 presents the sequences of the individual peptides; modified residues are indicated by underlining.

Table 6		
5/15 Modified Ara h 2 Peptides		
PEPTIDE NO	SEQ ID NO:	SEQUENCE
1		LTILVALALFLLAAH
2		ALALFLLAAHASARQ

5	3	LLAAHASARQQ <u>A</u> ELQ
	4	ASARQQ <u>A</u> ELQGDRRC
	5	QQ <u>A</u> ELQGDRRCQSQL <u>A</u>
	6	QGDRRCQSQLAR <u>A</u> NLR
	7	QSQLARANLR <u>A</u> CE <u>A</u> H
10	8	RANLR <u>A</u> CE <u>A</u> HLMQKI
	9	<u>A</u> CE <u>A</u> HLMQKIQA <u>D</u> ED
	10	LMQKIQA <u>D</u> EDSYERA <u>A</u>
	11	QA <u>D</u> EDSYERA <u>P</u> Y <u>S</u> P <u>S</u>
	12	SYERA <u>P</u> Y <u>S</u> P <u>S</u> QA <u>P</u> Y <u>S</u>
15	13	PY <u>S</u> P <u>S</u> QA <u>P</u> Y <u>S</u> SPY <u>D</u>
	14	QA <u>P</u> Y <u>S</u> SPY <u>D</u> RRGAG
	15	PSPY <u>D</u> RRGAGSSQH <u>Q</u>
	16	RRGAGSSQH <u>Q</u> ERCCN
	17	SSQH <u>Q</u> ERCCNQQE <u>Q</u> Q
20	18	ERCCNQQE <u>Q</u> QFKREA <u>A</u>
	19	QQE <u>Q</u> QFKREA <u>R</u> NLPQ
	20	FKREA <u>R</u> NLPQQCGLR
	21	RNLPQQCGLRAPQRC
	22	QCGLRAPQRCD <u>A</u> DVE
25	23	APQRCD <u>A</u> DVESGGRD
	24	D <u>A</u> DVESGGRDRY

25 *5/20 Native, Depleted for ≥ 2 Human Sites*

One strategy for reducing the effective IgE binding activity of a collection of overlapping Ara h 2 peptides is to remove from the collection those peptide that include two or more IgE binding sites, and therefore have the ability to cross-link anti-Ara h 2 IgE molecules. Individual peptides could be tested for their ability to simultaneously bind to two or more IgE molecules could be identified by direct

testing of IgE binding and/or cross-linking (e.g., histamine release). However, in the present Example, we simply designate those peptides that contain two complete IgE binding sites as determined by sequence analysis alone, relying on the above-described analyses to define the IgE binding sites. Under this analysis, peptides 3, 5, and 12 from Table 5 should be removed from the collection.

5/20 Native, Depleted for Immunodominant Epitopes

As mentioned above, human epitopes (6) and (7) (or mouse epitopes (5) and (6)) together are responsible for more than 40-50% of the IgE binding activity observed when human sera are tested against a panel of overlapping Ara h 2 peptides (see Stanley et al., *Arch. Biochem. Biophys.* 342:244-253, 1997, incorporated herein by reference). In certain embodiments of the invention, all peptides containing part or all of these sequences are removed from the 5/20 collection discussed above, to produce a 5/20 collection depleted of major immunodominant epitopes. That is, peptides 11-14, corresponding to amino acids 51-85, are removed from the collection. Interestingly, these peptides are not particularly active at stimulating T cell proliferation.

5/20 Native, Depleted for any Intact Human Sites

In yet another embodiment of the invention, the above-described 5/20 collection of native Ara h 2 peptides is depleted for those peptides that contain an intact IgE binding site as defined above in Example 3. Such depletion removes peptides 2-13 and 22-28 from the collection.

Example 4

Desensitization of PN-Sensitized Mice Using Ara h 2 Peptides

Introduction

This Example describes the use of a collection of antigen fragments (of the Ara h 2 protein) to desensitize individuals to peanut allergy. The Example also shows desensitization using a modified Ara h 2 protein whose IgE binding sites have been disrupted. The results with modified protein antigen are readily generalizable to peptide fragments, as described herein.

Materials and Methods

MICE AND REAGENTS: Female C3H/HeJ mice, 5-6 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained on PN-free chow, under specific pathogen-free conditions. Standard guidelines for the care and use of animals was followed.

Ara h 2 protein was purified as described by Burks et al. (*J. Allergy Clin. Immunol.* 8:172-179, 1992, incorporated herein by reference). Modified Ara h 2 was prepared as described in USSN 09/141,220 filed August 27, 1998, incorporated herein by reference. The sequence of the modified Ara h 2 differed from that of natural Ara h 2 (altered positions are underlined). The Ara h 2 peptide collection was the 5/20 collection described above in Example 4.

SENSITIZATION: Mice were sensitized by ig feeding with 5 mg of Ara h 2 plus 0.3 µg/gm body weight of cholera toxin (CT) as an adjuvant and were boosted twice, at weeks 1 and 3. Intragastric feeding was performed by means of a stainless steel blunt feeding needle as described by Li et al., *J. Allergy Clin. Immunol.* 103:206, 1999, incorporated herein by reference). Control mice received either CT alone or sham treatment.

DESENSITIZATION: Two weeks after sensitization, mice were treated with intranasal or subcutaneous peptide mix (either 2 µg or 20 µg), or with intranasal modified Ara h 2 (2 µg). One set of control mice was treated with intranasal wild type Ara h 2; another set was mock treated.

CHALLENGE: Two weeks later, desensitized mice were challenged orally with 5 mg of wild type Ara h 2, divided into two doses of 2.5 mg 30 min apart.

ASSAYS: Hypersensitivity testing and IgE measurement were performed as described above in Example 2. Plasma histamine levels and airway responsiveness were also assayed, as were Ara h 2-specific IgE and IgG2 levels.

RECHALLENGE: The mice that were sensitized, desensitized, and challenged as described above in Example were rechallenged with Ara h 2 protein 3 weeks later.

Results

Anti-Ara h 2 IgE levels in mice exposed to native Ara h 2 rose four fold during the "desensitization period". By contrast, these IgE levels did not increase

significantly in mice exposed to low or high dose peptides, and actually decreased almost two-fold in mice exposed to modified Ara h 2. Moreover, significant protection from anaphylaxis was observed with both the high dose peptides and the modified protein. In order to determine whether this protection were long term, we rechallenged the mice several (three) weeks later. As shown below in Table 7, the observed protection was long term:

Table 7		
"VACCINE"	ANTI ARA H 1 IgE LEVELS DURING 3 WEEKS BETWEEN CHALLENGES	SEVERITY OF ANAPHYLACTIC SYMPTOMS AS COMPARED WITH FIRST CHALLENGE
sham	increased	worse
low [native peptides] 20mers, 5aa stagger 2 µg/mouse	increased	worse
high [native peptides] 20mers, 5aa stagger 20 µg/mouse	no increase	no change
modified protein	modest increase	no significant change

These results clearly demonstrate that a collection of Ara h 2 peptides containing substantially all of the structural features of Ara h 2, can desensitize individuals allergic to Ara h 2. A modified Ara h 2 protein can have "similar effect, indicating that peptide collections lacking one or more effective IgE binding sites should also be useful desensitization tools.

Appendix

	ALLERGEN SOURCE	SYSTEMATIC AND ORIGINAL NAMES	MW kDa	SEQUENCE DATA	ACCESSION NO. OR REFERENCES
	WEED POLLENS				
5	<i>Asterales</i>				
	Ambrosia artemisiifolia (short ragweed)	Amb a 1: antigen E	38	C	8.20
		Amb a 2: antigen K	38	C	8.21
		Amb a 3: Ra3	11	C	22
		Amb a 5: Ra5	5	C	11.23
		Amb a 6: Ra6	10	C	24.25
		Amb a 7: Ra7	12	P	26
		Amb a ?	11	C	27
10	Ambrosia trifida (giant ragweed)	Amb t 5: Ra5G	4.4	C	9.10.28
	Artemisia vulgaris (mugwort)	Art v 1	27-29	C	28A
		Art v 2	35	P	29
	Helianthus annuus (sunflower)	Hel a 1	34	-	29a
		Hel a 2: profilin	15.7	C	Y15210
15	Mercurialis annua	Mer a 1: profilin	14-15	C	Y13271
	GRASS POLLENS				
	<i>Poales</i>				
	Cynodon dactylon (Bermuda grass)	Cyn d 1	32	C	30.S83343
		Cyn d 7		C	31.X91256
		Cyn d 12: profilin	14	C	31a.Y08390
20	Dactylis glomerata (orchard grass)	Dac g 1: AgDg1	32	P	32
		Dac g 2	11	C	33.S45354
		Dac g 3		C	33a.U25343
		Dac g 5	31	P	34
	Holcus lanatus (velvet grass)	Hol l 1		C	Z27084.Z6889 3

5	Lolium perenne (rye grass)	Lol p 1; group i	27	C	35.36
		Lol p 2; group II	11	C	37.37a.X7336
		Lol p 3; group III	11	C	3
		Lol p 5; Lol p IX.	31/35		38
		Lol p Ib			34.39
		Lol p 11; trypsin inh. Related	16		39a
10	Phalaris aquatica (canary grass)	Pha a 1		C	40.S80654
	Phleum pratense (timothy)	Phl p 1	27	C	X78813
		Phl p 2		C	41.X75925
		Phl p 4		P	41A
		Phl p 5; Ag25	32	C	42
		Phl p 6		C	43.Z27082
		Phl p 12; profilin		C	44.X77583
15	Poa pratensis (Kentucky blue grass)	Phl p 13; polygalacturonase	55-60	C	AJ238848
		Poa p 1; group I	33	P	46
		Poa p 5	31/34	C	34.47
20	Sorghum halepense (Johnson grass)	Sor h 1		C	48
	TREE POLLENS				
25	Fagales				
	Alnus glutinosa (alder)	Aln g 1	17	C	S50892
30	Betula verrucosa (birch)	Bet v 1	17	C	see list of isoallergens
		Bet v 2; profilin	15	C	M65179
		Bet v 3	8	C	X79267
		Bet v 4		C	X87153/S548
		Bet v 5; isoflavone reductase homologue	33.5	C	19 AF135127
30	Carpinus betulus (hornbeam)	Car b 1	17	C	51

5	Castanea sativa (chestnut)	Cas s 1: Bet v 1 homologue	22	P	52
	Corylus avelana (hazel)	Cor a 1	17	C	53
	Quercus alba (white oak)	Que a 1	17	P	54
	Cryptomeria japonica (sugi)	Cry j 1 Cry j 2	41-45	C C	55.56 57, D29772
10	Juniperus ashei (mountain cedar)	Jun a 1	43	P	P81294
		Jun a 3	30	P	P81295
15	Juniperus oxycedrus (prickly juniper)	Jun o 2; calmodulin-like	29	C	AF031471
	Juniperus sabinoides (mountain cedar)	Jun s 1	50	P	58
	Juniperus virginiana (eastern red cedar)	Jun v 1	43	P	P81825
20	Oleales:				
	Fraxinus excelsior (ash)	Fra e 1	20	P	58A
	Ligustrum vulgare (privet)	Lig v 1	20	P	58A
25	Olea europea (olive)	Ole e 1;	16	C	59.60
		Ole e 2: profilin	15-18	C	60A
		Ole e 3;	9.2		60B
		Ole e 4;	32	P	P80741
		Ole e 5: superoxide dismutase	16 10	P C	P80740 U86342
		Ole e 6;			
30	Syringa vulgaris (lilac)	Syr v 1:	20	P	58A
	MITES				
	Acarus siro (mite)	Aca s 13: fatty acid-bind.prot.	14*	C	AJ006774

5

Blomia tropicalis (mite)	Blo t 5:		C	U59102
	Blo t 12: Btl 1a		C	U27479
	Blo t 13: Bt6 fatty acid-binding prot		C	U58106
Dermatophagoides pteronyssinus (mite)	Der p 1: antigen P1	25	C	61
	Der p 2:	14	C	62
	Der p 3: trypsin	28/30	C	63
	Der p 4: amylase	60	C	64
	Der p 5:	14	P	65
	Der p 6: chymotrypsin	25	C	66
	Der p 7:	22-28	C	
	Der p 8: glutathione transferase		P C	67 67A
	Der p 9: collagenolytic serine prot.			67B
	Der p 10: tropomyosin	36		Y14906
Dermatophagoides microceras (mite)	Der m 1;	25	P	68
Dermatophagoides farinae (mite)	Der f 1 :	25	C	69
	Der f 2 :	14	C	70.71
	Der f 3 :	30	C	63
	Der f 10; tropomyosin		C	72
Lepidoglyphus destructor (storage mite)	Lep d 2.0101:	15	C	73.74.75
	Lep d 2.0102;	15	C	75
ANIMALS				

5	Bos domesticus (domestic cattle) (see also foods)	Bos d 2: Ag β .lipocalin	20	C	76.L42867
		Bos d 4: alpha-lactalbumin	14.2	C	M18780
		Bos d 5: beta-lactoglobulin	18.3	C	X14712
		Bos d 6: serum albumin	67	C	M73993
		Bos d 7: immunoglobulin	160		77
		Bos d 8: caseins	20-30		77 Bos d 8: caseins
5	Canis familiaris (Canis domesticus (dog)	Can f 1:	25	C	78.79
		Can f 2:	27	C	78.79
		Can f 3: albumin		C	S72946
10	Equus caballus (domestic horse)	Equ c 1: lipocalin	25	C	U70823
		Equ c 2: lipocali	18.5	P	79A. 79B
10	Felis domesticus (cat saliva)	Fel d 1: cat-1	38	C	15
	Mus musculus (mouse urine)	Mus m 1: MUP	19	C	80.81
	Rattus norvegicus (rat urine)	Rat n 1	17	C	82.83
15	FUNGI				
	<i>Ascomycota</i>				
	Dothidiales				

Alternaria alternata	Alt a 1;	28	C	U82633
	Alt a 2;	25	C	
	Alt a 3; heat shock prot	70	C	U87807. U87808
	Alt a 6; ribosomal protein	11	C	X78222.
	Alt a 7; YCP4 protein	22	C	U87806
	Alt a 10; aldehyde dehydrogenase	53	C	X78225 X78227. P42041
Cladosporium herbarum	Cla h 1;	13		83a. 83b
	Cla h 2;	23		83a. 83b
	Cla h 3; aldehyde dehydrogenase	53	C	X78228
	Cla h 4; ribosomal protein	11	C	X78223
	Cla h 5; YCP4 protein	22	C	X78224
	Cla h 6; enolase	46	C	X78226
Eurotiales				
5	Asp fl 13; alkaline serine proteinase	34		84

Aspergillus	Asp f 1:	18	C	M83781.S393
Fumigatus	Asp f 2:	37	C	30
	Asp f 3: peroxisomal protein	19	C	U56938 U20722
	Asp f 4:	30	C	
	Asp f 5: metalloprotease	42	C	AJ001732 Z30424
	Asp f 6: Mn superoxide dismutase	26.5	C	U53561
	Asp f 7:	12		
	Asp f 8: ribosomal protein P2	11	C	AJ223315
	Asp f 9:	34	C	AJ224333
	Asp f 10: aspartic protease	34	C	AJ223327 X85092
	Asp f 11: peptidyl-prolyl isom	24		
	Asp f 12: heat shock prot. P70	65		84a
	Asp f 13: alkaline serine proteinase	34	C	U92465
	Asp f 15:	16		84b
	Asp f 16:	43	C	
	Asp f 17:		C	
	Asp f 18: vacuolar serine	34	C	AJ002026 g3643813
	Asp f ?:	90		AJ224865
	Asp f ?:	55	P	
			P	84c
				85
				86
Aspergillus niger	Asp n 14: beta-xylosidase	105	C	AF108944
	Asp n 18: vacuolar serine proteinase	34	C	84b
	Asp n ?:	85	C	Z84377

5

10

15

Aspergillus oryzae	Asp o 2: TAKA-amylase A	53	C	D00434.M332 18
	Asp o 13: alkaline serine proteinase	34	C	X17561
Penicillium brevicompactum	Pen b 13: alkaline serine Proteinase	33		86a
Penicillium citrinum	Pen c 1: heat shock prot. P70	70	C	U64207
	Pen c 3: peroxisomal membrane protein Pen c 13: alkaline serine proteinase	33		86b 86a
Penicillium notatum	Pen n 1: N-acetyl glucosaminidase	68		87
	Pen n 13: alkaline serine proteinase	34		89
	Pen n 18: vacuolar serine proteinase	32		89
Penicillium oxalicum	Pen o 18: vacuolar serine proteinase	34		89
Onygenales				
Trichophyton rubrum	Tri r 2:		C	90
	Tri r 4: serine protease		C	90
Trichophyton tonsurans	Tri t 1:	30	P	91
	Tri t 4: serine protease	83	C	90
Saccharomycetales				
Candida albicans	Cand a 1:	40	C	88
Candida boidinii	Cand b 2:	20	C	J04984. J04985
Basidiomycota				
Basidiolaelastomyces				

5	Malassezia furfur	Mal f 1: Mal f 2: MF1 peroxisomal membrane protein Mal f 3: MF2 peroxisomal membrane protein Mal f 4: Mal f 5: Mal f 6: cyclophilin homologue	21 20 35 18* 17*	C C C C	91a AB011804 AB011805 Takesako. p.c. AJ011955 AJ011956
	Basidiomycetes				
	Psilocybe cubensis	Psi c 1: Psi c 2: cyclophilin	16		91b
	Coprinus comatus (shaggy cap)	Cop c 1; Cop c 2:	11	C	AJ132235
	INSECTS				
	Apis mellifera (honey bee)	Api m 1: phospholipase A2 Api m 2: hyaluronidase Api m 4: melittin	16 44 3	C C C	92 93 94
	Bombus pennsylvanicus (bumble bee)	Bom p 1: phospholipase Bom p 4: protease	16	P P	95 95
	Blattella germanica (German cockroach)	Bla g 1: Bd90k Bla g 2: aspartic protease Bla g 4: calycin Bla g 5: glutathione transf. Bla g 6: troponin C	36 21 22 27	C C C C C	96 97 98 98
	Periplaneta americana (American cockroach)	Per a 1: Cr-P11 Per a 3: Cr-P1 Per a 7: tropomyosin	72-78 37	C C C	98A Y14854
	10				
15					

5

Chironomus thummi thummi (midges)	Chi t 1-9: hemoglobin	16	C	99
	Chi t 1.01: component III	16	C	P02229
	Chi t 1.02: component IV	16	C	P02230
	Chi t 2.0101: component I	16	C	P02221
	Chi t 2.0102: component IA	16	C	P02221
	Chi t 3: component II-beta	16	C	P02222
	Chi t 4: component IIIA	16	C	P02231
	Chi t 5: component VI	16	C	P02224
	Chi t 6.01: component VIIA	16	C	P02226
	Chi t 6.02: component IX	16	C	P02223
	Chi t 7: component VIIB	16	C	P02225
	Chi t 8: component VIII	16	C	P02227
	Chi t 9: component X	16	C	P02228
Dolichovespula maculata (white face hornet)	Dol m 1: phospholipase A1	35	C	100
	Dol m 2: hyaluronidase	44	C	101
	Dol m 5: antigen 5	23	C	102.103
Dolichovespula arenaria (yellow hornet)	Dol a 5: antigen 5	23	C	104

	Polistes annularis (wasp)	Pol a 1; phospholipase A1	35	P	105
		Pol a 2; hyaluronidase	44	P	105
		Pol a 5; antigen 5	23	C	104
5	Polistes dominulus (Mediterranean paper wasp)	Pol d 1;	32-34	C	DR Hoffman
		Pol d 4; serine protease			DR Hoffman
		Pol d 5;			P81656
	Polistes exclamans (wasp)	Pol e 1; phospholipase A1	34	P	107
		Pol e 5; antigen 5	23	C	104
		Pol f 5; antigen 5	23	C	106
10	Polistes metricus (wasp)	Pol m 5; antigen 5	23	P	106
		Vesp c 1; phospholipase	34	P	107
		Vesp c 5.0101; antigen 5	23	C	106
	Vespa crabo (European hornet)	Vesp c 5.0102; antigen 5	23	C	106
		Vesp m 1.01;			DR Hoffman
		Vesp m 1.02;			DR Hoffman
15	Vespa mandarina (giant asian hornet)	Vesp m 5;			P81657
		Ves f 5; antigen 5	23	C	106
		Ves g 5; antigen 5	23	C	106
20	Vespula flavopilosa (yellowjacket)	Ves m 1; phospholipase A1	33.5	C	108
		Ves m 2; hyaluronidase	44	P	109
		Ves m 5; antigen 5	23	23	104

5	Vespula pennsylvanica (yellowjacket)	Ves p 5; antigen 5	23	C	106
	Vespula squamosa (yellowjacket)	Ves s 5; antigen 5	23	C	106
	Vespula vidua (wasp)	Ves vi 5;	23	C	106
	Vespula vulgaris (yellowjacket)	Ves v 1; phopholipase A1	35	C	105A
Ves v 2; hyaluronidase		44	P	105A	
Ves v 5; antigen 5		23	C	104	
10	Myrmecia pilosula (Australian jumper ant)	Myr p 1;		C	X70256
		Myr p 2;		C	S81785
15	Solenopsis geminata (tropical fire ant)	Sol g 2; Sol g 4			DR Hoffman DR Hoffman
	Solenopsis invicta (fire ant)	Sol i 2;	13	C	110.111
		Sol i 3;	24	C	110
		Soli 4;	13	C	110
20	Solenopsis saevissima (brazilian fire ant)	Sols 2;			DR Hoffman
	FOODS				
	Gadus callarias (cod)	Gad c 1; allergen M	12	C	112.113
	Salmo salar (Atlantic salmon)	Sals 1; parvalbumin	12	C	X97824 X97825

	Bos domesticus (domestic cattle)	Bos d 4: alpha-lactalbumin	14.2	C	M18780
		Bos d 5: beta-lactoglobulin	18.3	C	X14712
		Bos d 6: serum albumin	67	C	M73993
		Bos d 7: immunoglobulin	160		77
		Bos d 8: caseins	20-30		77
5	Gallus domesticus (chicken)	Gal d 1: ovomucoid	28	C	114.115
		Gald 2: ovalbumin	44	C	114.115
		Gald 3: conalbumin (Ag22)	78	C	114.115
		Gald 4: lysozyme	14	C	114.115
10	Metapenaeus ensis (shrimp)	Met e 1: tropomyosin		C	U08008
	Penaeus aztecus (shrimp)	Pen a 1: tropomyosin	36	P	116
	Penaeus indicus (shrimp)	Pen i 1: tropomyosin	34	C	117
	Todarodes pacificus (squid)	Tod p 1: tropomyosin	38	P	117A
15	Halotis Midae (abalone)	Hal m 1	49	-	117B
	Apium graveolens (celery)	Api g 1: Bet v 1 homologue	16*	C	Z48967
	Brassica juncea (oriental mustard)	Bra j 1: 2S albumin	14	C	118
	Brassica rapa (turnip)	Bra r 2: prohevein-like protein	25	?	P81729
20	Hordeum vulgare (barley)	Hor v 1: BMAI-1	15	C	119
	Malus domestica (apple)	Mal d 1: Bet v 1 homologue		C	X83672
		Mal d 3: lipid transfer protein	9	C	Pastorello

5	Oryza sativa (rice)	Ory s 1;		C	U31771
	Persea americana (avocado)	Pers a 1; endochitinase	32	C	Z78202
	Prunus armeniaca (apricot)	Pru ar 1; Bet v 1 homologue Pru ar 3; lipid transfer protein	9	P	U93165
	Prunus avium (sweet cherry)	Pru av 1; Bet v 1 homologue Pru av 2; thaumatin homologue		C C	U66076 U32440
	Prunus persica (peach)	Pru p 3; lipid transfer protein	10	P	P81402
10	Sinapis alba (yellow mustard)	Sin a 1; 2S albumin	14	C	120
	Glycine max (soybean)	Gly m 1.0101; HPS Gly m 1.0102; HPS Gly m 2 Gly m 3; profilin	7.5 7 8 14	P P P C	121 121 A57106 AJ223982
	Arachis hypogaea (peanut)	Ara h 1; vicilin Ara h 2; conglutin Ara h 3; glycinin Ara h 4; glycinin Ara h 5; profilin Ara h 6; conglutin homolog Ara h 7; conglutin homolog	63.5 17 14 37 15 15 15	C C C C C C C	L34402 L77197 AF093541 AF086821 AF059616 AF092846 AF091737
	Actinidia chinensis (kiwi)	Act c 1; cysteine protease	30	P	P00785
20	Solanum tuberosum (potato)	Sol t 1; patatin	43	P	P15476

5	Bertholletia excelsa (Brazil nut)	Ber e 1: 2S albumin	9	C	P04403.M171 46
	Juglans regia (English walnut)	Jug r 1: 2S albumin Jug r 2: vicilin	44	C C	U66866 AF066055
	Ricinus communis (Castor bean)	Ric c 1: 2S albumin		C	P01089
10	OTHERS				
	Ascaris suum (worm)	Asc s 1:	10	P	122
	Aedes aegyptii (mosquito)	Aed a 1: apyrase Aed a 2:	68 37	C C	L12389 M33157

Hevea brasiliensis (rubber)	Hev b 1: elongation factor	58	P	123.124
	Hev b 2: (1.3-glucanase	58	P	123.124
	Hev b 2: (1.3-glucanase	34/36	C	125
	Hev b 3	24	P	126.127
	Hev b 4: component of microhelix protein complex	100/110/1 15	P	128
	Hev b 5		C	U42640
	Hev b 6.01 hevein precursor	16	C	
	Hev b 6.02 hevein			M36986/p028
	Hev b 6.03 C-terminal fragment	20	C C	77
	Hev b 7: patatin homologue	5 14	C	M36986/p028 77
	Hev b 8: profilin			
	Hev b 9: enolase	46		M36986/p028
			C	77
			C	
		14		U80598
		51		
				Y15042 AJ132580/AJ1 32581

- 5 1. Marsh, D.G., and L.R. Freidhoff. 1992. ALBE. an allergen database. IUIS, Baltimore, MD. Edition 1.0.
2. Marsh, D. G., L. Goodfriend, T. P. King, H. Lowenstein, and T. A. E. Platts-Mills. 1986. Allergen nomenclature. Bull WHO 64:767-770.
- 10 3. King, T.P., P.S. Norman, and J.T. Cornell. 1964. Isolation and characterization of allergen from ragweed pollen. II. Biochemistry 3:458-468.
4. Lowenstein, H. 1980. Timothy pollen allergens. Allergy 35:188-191.
5. Aukrust, L. 1980. Purification of allergens in Cladosporium herbarum. Allergy 35:206-207.
6. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54:61-75.

7. Bodmer, J. G., E. D. Albert, W. F. Bodmer, B. Dupont, H. A. Erlich, B. Mach, S. G. E. Marsh, W. R. Mayr, P. Parham, T. Sasuki, G. M. Th. Schreuder, J. L. Strominger, A. Svejgaard, and P. I. Terasaki. 1991. Nomenclature for factors of the HLA system, 1990. *Immunogenetics* 33:301-309.
8. Griffith, I.J., J. Pollock, D.G. Klapper, B.L. Rogers, and A.K. Nault. 1991. Sequence polymorphism of Amb a I and Amb a II, the major allergens in *Ambrosia artemisiifolia* (short ragweed). *Int. Arch. Allergy Appl. Immunol.* 96:296-304.
9. Roebber, M., D. G. Klapper, L. Goodfriend, W. B. Bias, S. H. Hsu, and D. G. Marsh. 1985. Immunochemical and genetic studies of Amb t V (Ra5G), an Ra5 homologue from giant ragweed pollen. *J. Immunol.* 134:3062-3069.
10. Metzler, W. J., K. Valentine, M. Roebber, M. Friedrichs, D. G. Marsh, and L. Mueller. 1992. Solution structures of ragweed allergen Amb t V. *Biochemistry* 31:5117-5127.
11. Metzler, W. J., K. Valentine, M. Roebber, D. G. Marsh, and L. Mueller. 1992. Proton resonance assignments and three-dimensional solution structure of the ragweed allergen Amb a V by nuclear magnetic resonance spectroscopy. *Biochemistry* 31:8697-8705.
12. Goodfriend, L., A.M. Choudhury, J. Del Carpio, and T.P. King. 1979. Cytochromes C: New ragweed pollen allergens. *Fed. Proc.* 38:1415.
13. Ekramoddoullah, A. K. M., F. T. Kisil, and A. H. Sehon. 1982. Allergenic cross reactivity of cytochrome c from Kentucky bluegrass and perennial ryegrass pollens. *Mol. Immunol.* 19:1527-1534.
14. Ansari, A. A., E. A. Killoran, and D. G. Marsh. 1987. An investigation of human response to perennial ryegrass (*Lolium perenne*) pollen cytochrome c (Lol p X). *J. Allergy Clin. Immunol.* 80:229-235.
15. Morgenstern, J.P., I.J. Griffith, A.W. Brauer, B.L. Rogers, J.F. Bond, M.D. Chapman, and M. Kuo. 1991. Amino acid sequence of Fel d I, the major allergen of the domestic cat: protein sequence analysis and cDNA cloning. *Proc. Natl. Acad. Sci. USA* 88:9690-9694.
16. Griffith, I.J., S. Craig, J. Pollock, X. Yu, J.P. Morgenstern, and B.L. Rogers. 1992. Expression and genomic structure of the genes encoding FdI, the major allergen from the domestic cat. *Gene* 113:263-268.
17. Weber, A., L. Marz, and F. Altmann. 1986. Characteristics of the asparagine-linked oligosaccharide from honey-bee venom phospholipase A2. *Comp. Biochem. Physiol.* 83B:321-324.
18. Weber, A., H. Schroder, K. Thalberg, and L. Marz. 1987. Specific interaction of IgE antibodies with a carbohydrate epitope of honey bee venom phospholipase A2. *Allergy* 42:464-470.
19. Stanworth, D. R., K. J. Dorrington, T. E. Hugli, K. Reid, and M. W. Turner. 1990. Nomenclature for synthetic peptides representative of immunoglobulin chain sequences. *Bulletin WHO* 68:109-111.
20. Rafnar, T., I. J. Griffith, M. C. Kuo, J. F. Bond, B. L. Rogers, and D.G. Klapper. 1991. Cloning of Amb a I (Antigen E), the major allergen family of short ragweed pollen. *J. Biol. Chem.* 266:1229-1236.
21. Rogers, B.L., J.P. Morgenstern, I.J. Griffith, X.B. Yu, C.M. Counsell, A.W. Brauer, T.P. King, R.D. Garman, and M.C. Kuo. 1991. Complete sequence of the allergen Amb a II: recombinant expression and reactivity with T cells from ragweed allergic patients. *J. Immunol.* 147:2547-2552.
22. Klapper, D.G., L. Goodfriend, and J.D. Capra. 1980. Amino acid sequence of ragweed allergen Ra3. *Biochemistry* 19:5729-5734.
23. Ghosh, B., M.P. Perry, T. Rafnar, and D.G. Marsh. 1993. Cloning and expression of immunologically active recombinant Amb a V allergen of short ragweed (*Ambrosia artemisiifolia*) pollen. *J. Immunol.* 150:5391-5399.
24. Roebber, M., R. Hussain, D. G. Klapper, and D. G. Marsh. 1983. Isolation and properties of a new short ragweed pollen allergen, Ra6. *J. Immunol.* 131:706-711.
25. Lubahn, B., and D.G. Klapper. 1993. Cloning and characterization of ragweed allergen Amb a VI (abst). *J. Allergy Clin. Immunol.* 91:338.
26. Roebber, M., and D.G. Marsh. 1991. Isolation and characterization of allergen Amb a VII from short ragweed pollen. *J. Allergy Clin. Immunol.* 87:324.
27. Rogers, B.L., J. Pollock, D.G. Klapper, and I.J. Griffith. 1993. Cloning, complete sequence, and recombinant expression of a novel allergen from short ragweed pollen (abst). *J. Allergy Clin. Immunol.* 91:339.
28. Goodfriend, L., A.M. Choudhury, D.G. Klapper, K.M. Coulter, G. Dorval, J. DelCarpio, and C.K. Osterland. 1985. Ra5G, a homologue of Ra5 in giant ragweed pollen: isolation. HLA-DR-associated activity and amino acid sequence. *Mol. Immunol.* 22:899-906.
- 28A. Breitenbach M, pers. comm.

29. Nilsen, B. M., K. Sletten, M. O'Neill, B. Smestad Paulsen, and H. van Halbeek. 1991. Structural analysis of the glycoprotein allergen Art v II from pollen of mugwort (*Artemisia vulgaris*). *J. Biol. Chem.* 266:2660-2668.
- 5 29A Jimenez A, Moreno C, Martinez J, Martinez A, Bartolome B, Guerra F, Palacios R 1994. Sensitization to sunflower pollen: only an occupational allergy? *Int Arch Allergy Immunol* 105:297-307.
30. Smith, P.M., Suphioglu, C., Griffith, I.J., Theriault, K., Knox, R.B. and Singh, M.B. 1996. Cloning and expression in yeast *Pichia pastoris* of a biologically active form of Cyn d 1, the major allergen of Bermuda grass pollen. *J. Allergy Clin. Immunol.* 98:331-343.
- 10 31. Suphioglu, C., Ferreira, F. and Knox, R.B. 1997. Molecular cloning and immunological characterisation of Cyn d 7, a novel calcium-binding allergen from Bermuda grass pollen. *FEBS Lett.* 402:167-172.
- 31a. Asturias JA, Arilla MC, Gomez-Bayon N, Martinez J, Martinez A, and Palacios R. 1997. Cloning and high level expression of *Cynodon dactylon* (Bermuda grass) pollen profilin (Cyn d 12) in *Escherichia coli*: purification and characterization of the allergen. *Clin Exp Allergy* 27:1307-1313.
- 15 32. Mecheri, S., G. Peltre, and B. David. 1985. Purification and characterization of a major allergen from *Dactylis glomerata* pollen: The Ag Dg 1. *Int. Arch. Allergy Appl. Immunol.* 78:283-289.
33. Roberts, A.M., L.J. Bevan, P.S. Flora, I. Jepson, and M.R. Walker. 1993. Nucleotide sequence of cDNA encoding the Group II allergen of Cocksfoot/Orchard grass (*Dactylis glomerata*), Dac g II. *Allergy* 48:615-623.
- 20 33a. Guerin-Marchand, C., Senechal, H., Bouin, A.P., Leduc-Brodard, V., Taudou, G., Weyer, A., Peltre, G. and David, B. 1996. Cloning, sequencing and immunological characterization of Dac g 3, a major allergen from *Dactylis glomerata* pollen. *Mol. Immunol.* 33:797-806.
34. Klysner, S., K. Welinder, H. Lowenstein, and F. Matthiesen. 1992. Group V allergens in grass pollen IV. Similarities in amino acid compositions and amino terminal sequences of the group V allergens from *Lolium perenne*, *Poa pratensis* and *Dactylis glomerata*. *Clin. Exp. Allergy* 22: 491-497.
- 25 35. Perez, M., G. Y. Ishioka, L. E. Walker, and R. W. Chesnut. 1990. cDNA cloning and immunological characterization of the rye grass allergen Lol p I. *J. Biol. Chem.* 265:16210-16215.
36. Griffith, I. J., P. M. Smith, J. Pollock, P. Theerakulpisut, A. Avjioglu, S. Davies, T. Hough, M. B. Singh, R. J. Simpson, L. D. Ward, and R. B. Knox. 1991. Cloning and sequencing of Lol p I, the major allergenic protein of rye-grass pollen. *FEBS Letters* 279:210-215.
- 30 37. Ansari, A. A., P. Shenbagamurthi, and D.G. Marsh. 1989. Complete amino acid sequence of a *Lolium perenne* (perennial rye grass) pollen allergen, Lol p II. *J. Biol. Chem.* 264:11181-11185.
- 37a. Sidoli, A., Tamborini, E., Giuntini, L., Levi, S., Volonte, G., Paini, C., De Lalla, C., Siccardi, A.G., Baralle, F.E., Galliani, S. and Arosio, P. 1993. Cloning, expression, and immunological characterization of recombinant *Lolium perenne* allergen Lol p II. *J. Biol. Chem.* 268:21819-21825.
- 35 38. Ansari, A. A., P. Shenbagamurthi, and D. G. Marsh. 1989. Complete primary structure of a *Lolium perenne* (perennial rye grass) pollen allergen, Lol p III: Comparison with known Lol p I and II sequences. *Biochemistry* 28:8665-8670.
- 40 39. Singh, M. B., T. Hough, P. Theerakulpisut, A. Avjioglu, S. Davies, P. M. Smith, P. Taylor, R. J. Simpson, L. D. Ward, J. McCluskey, R. Puy, and R.B. Knox. 1991. Isolation of cDNA encoding a newly identified major allergenic protein of rye-grass pollen: Intracellular targeting to the amyloplast. *Proc. Natl. Acad. Sci.* 88:1384-1388.
- 39a. van Ree R, Hoffman DR, van Dijk W, Brodard V, Mahieu K, Koeleman CA, Grande M, van Leeuwen WA, Aalberse RC. 1995. Lol p XI, a new major grass pollen allergen, is a member of a family of soybean trypsin inhibitor-related proteins. *J Allergy Clin Immunol* 95:970-978.
- 45 40. Suphioglu, C. and Singh, M.B. 1995. Cloning, sequencing and expression in *Escherichia coli* of Pha a I and four isoforms of Pha a 5, the major allergens of canary grass pollen. *Clin. Exp. Allergy* 25:853-865.
- 50 41. Dolecek, C., Vrtala, S., Laffer, S., Steinberger, P., Kraft, D., Scheiner, O. and Valenta, R. 1993. Molecular characterization of Phl p II, a major timothy grass (*Phleum pratense*) pollen allergen. *FEBS Lett.* 335:299-304.
- 41A. Fischer S, Grote M, Fahlbusch B, Muller WD, Kraft D, Valenta R. 1996. Characterization of Phl p 4, a major timothy grass (*Phleum pratense*) pollen allergen. *J Allergy Clin Immunol* 98:189-198.
- 55 42. Matthiesen, F., and H. Lowenstein. 1991. Group V allergens in grass pollens. I. Purification and characterization of the group V allergen from *Phleum pratense* pollen. Phl p V. *Clin. Exp. Allergy* 21:297-307.

43. Petersen.A., Bufer.A., Schramm.G., Schlaak.M. and Becker.W.M. 1995. Characterization of the allergen group VI in timothy grass pollen (Phl p 6). II. cDNA cloning of Phl p 6 and structural comparison to grass group V. *Int. Arch. Allergy Immunol.* 108:55-59.
- 5 44. Valenta.R., Ball.T., Vrtala.S., Duchene.M., Kraft.D. and Scheiner.O. 1994. cDNA cloning and expression of timothy grass (*Phleum pratense*) pollen profilin in *Escherichia coli*: comparison with birch pollen profilin. *Biochem. Biophys. Res. Commun.* 199:106-118.
46. Esch, R. E., and D. G. Klapper. 1989. Isolation and characterization of a major cross-reactive grass group I allergenic determinant. *Mol. Immunol.* 26:557-561.
47. Olsen, E., L. Zhang, R. D. Hill, F. T. Kisil, A. H. Schon, and S. Mohapatra. 1991. Identification and characterization of the Poa p IX group of basic allergens of Kentucky bluegrass pollen. *J. Immunol.* 147:205-211.
- 10 48. Avjioglu, A., M. Singh, and R.B. Knox. 1993. Sequence analysis of Sor h I, the group I allergen of Johnson grass pollen and its comparison to rye-grass Lol p I (abst). *J. Allergy Clin. Immunol.* 91:340.
51. Larsen, J.N., P. Strøman, and H. Ipsen. 1992. PCR based cloning and sequencing of isogenes encoding the tree pollen major allergen Car b I from *Carpinus betulus*, hornbeam. *Mol. Immunol.* 29:703-711.
52. Kos T, Hoffmann-Sommergruber K, Ferreira F, Hirschwehr R, Ahorn H, Horak F, Jäger S, Spert W, Kraft D, Scheiner O. 1993. Purification, characterization and N-terminal amino acid sequence of a new major allergen from European chestnut pollen--Cas s I. *Biochem Biophys Res Commun* 196:1086-92.
- 20 53. Breiteneder, H., F. Ferreira, K. Hoffman-Sommergruber, C. Ebner, M. Breitenbach, H. Rumpold, D. Kraft, and O. Scheiner. 1993. Four recombinant isoforms of Cor a I, the major allergen of hazel pollen. *Europ. J. Biochem.* 212:355-362.
54. Ipsen, H., and B.C. Hansen. 1991. The NH2-terminal amino acid sequence of the immunochemically partial identical major allergens of alder (*Alnus glutinosa*) Aln g I, birch (*Betula verrucosa*) Bet v I, hornbeam (*Carpinus betulus*) Car b I and oak (*Quercus alba*) Que a I pollens. *Mol. Immunol.* 28:1279-1288.
- 25 55. Tanai, M., S. Ando, M. Usui, M. Kurimoto, M. Sakaguchi, S. Inouye, and T. Matuhasi. 1988. N-terminal amino acid sequence of a major allergen of Japanese cedar pollen (Cry j I). *FEBS Lett.* 239:329-332.
- 30 56. Griffith, I.J., A. Lussier, R. Garman, R. Koury, H. Yeung, and J. Pollock. 1993. The cDNA cloning of Cry j I, the major allergen of *Cryptomeria japonica* (Japanese cedar) (abst). *J. Allergy Clin. Immunol.* 91:339.
57. Sakaguchi, M., S. Inouye, M. Tanai, S. Ando, M. Usui, and T. Matuhasi. 1990. Identification of the second major allergen of Japanese cedar pollen. *Allergy* 45:309-312.
- 35 58. Gross GN, Zimburean JM, Capra JD 1978. Isolation and partial characterization of the allergen in mountain cedar pollen. *Scand J Immunol* 8:437-41
- 58A Obispo TM, Melero JA, Carpizo JA, Carreira J, Lombardero M 1993. The main allergen of *Olea europaea* (Ole e I) is also present in other species of the oleaceae family. *Clin Exp Allergy* 23:311-316.
- 40 59. Cardaba, B., D. Hernandez, E. Martin, B. de Andres, V. del Pozo, S. Gallardo, J.C. Fernandez, R. Rodriguez, M. Villalba, P. Palomino, A. Basomba, and C. Lahoz. 1993. Antibody response to olive pollen antigens: association between HLA class II genes and IgE response to Ole e I (abst). *J. Allergy Clin. Immunol.* 91:338.
60. Villalba, M., E. Batanero, C. Lopez-Otin, L.M. Sanchez, R.I. Monsalve, M.A. Gonzalez de la Pena, C. Lahoz, and R. Rodriguez. 1993. Amino acid sequence of Ole e I, the major allergen from olive tree pollen (*Olea europaea*). *Europ.J. Biochem.* 216:863-869.
- 45 60A. Asturias JA, Arilla MC, Gomez-Bayon N, Martinez J, Martinez A, Palacios R 1997. Cloning and expression of the panallergen profilin and the major allergen (Ole e I) from olive tree pollen. *J Allergy Clin Immunol* 100:365-372.
- 50 60B. Batanero E, Villalba M, Ledesma A, Puente XS, Rodriguez R. 1996. Ole e 3, an olive-tree allergen, belongs to a widespread family of pollen proteins. *Eur J Biochem* 241: 772-778.
61. Chua, K. Y., G. A. Stewart, and W. R. Thomas. 1988. Sequence analysis of cDNA encoding for a major house dust mite allergen, Der p I. *J. Exp. Med.* 167:175-182.
62. Chua, K. Y., C. R. Doyle, R. J. Simpson, K. J. Turner, G. A. Stewart, and W. R. Thomas. 1990. Isolation of cDNA coding for the major mite allergen Der p II by IgE plaque immunoassay. *Int. Arch. Allergy Appl. Immunol.* 91:118-123.
- 55

63. Smith WA, Thomas WR. 1996. Comparative analysis of the genes encoding group 3 allergens from *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*. *Int Arch Allergy Immunol* 109: 133-40.
- 5 64. Lake, F.R., L.D. Ward, R.J. Simpson, P.J. Thompson, and G.A. Stewart. 1991. House dust mite-derived amylase: Allergenicity and physicochemical characterisation. *J. Allergy Clin. Immunol.* 87:1035-1042.
65. Tovey, E. R., M. C. Johnson, A. L. Roche, G. S. Cobon, and B. A. Baldo. 1989. Cloning and sequencing of a cDNA expressing a recombinant house dust mite protein that binds human IgE and corresponds to an important low molecular weight allergen. *J. Exp. Med.* 170:1457-1462.
- 10 66. Yasueda, H., T. Shida, T. Ando, S. Sugiyama, and H. Yamakawa. 1991. Allergenic and proteolytic properties of fourth allergens from *Dermatophagoides* mites. In: "Dust Mite Allergens and Asthma. Report of the 2nd international workshop" A. Todt, Ed., UCB Institute of Allergy, Brussels, Belgium, pp. 63-64.
- 15 67. Shen, H.-D., K.-Y. Chua, K.-L. Lin, K.-H. Hsieh, and W.R. Thomas. 1993. Molecular cloning of a house dust mite allergen with common antibody binding specificities with multiple components in mite extracts. *Clin. Exp. Allergy* 23:934-40.
- 67A. O'Neil GM, Donovan GR, Baldo BA. 1994. Cloning and charaterisation of a major allergen of the house dust mite *Dermatophagoides pteronyssinus*, homologous with glutathione S-transferase. *Biochim Biophys Acta*.1219:521-528.
- 20 67B. King C, Simpson RJ, Moritz RL, Reed GE, Thompson PJ, Stewart GA. 1996. The isolation and characterization of a novel collagenolytic serine protease allergen (Der p 9) from the dust mite *Dermatophagoides pteronyssinus*. *J Allergy Clin Immunol* 98:739-47.
68. Lind P, Hansen OC, Horn N. 1988. The binding of mouse hybridoma and human IgE antibodies to the major fecal allergen, Der p I of *D. pteronyssinus*. *J. Immunol.* 140:4256-4262.
- 25 69. Dilworth, R. J., K. Y. Chua, and W. R. Thomas. 1991. Sequence analysis of cDNA coding for a major house dust allergen Der f I. *Clin. Exp. Allergy* 21:25-32.
70. Nishiyama, C., T. Yunki, T. Takai, Y. Okumura, and H. Okudaira. 1993. Determination of three disulfide bonds in a major house dust mite allergen, Der f II. *Int. Arch. Allergy Immunol.* 101:159-166.
71. Trudinger, M., K. Y. Chua, and W. R. Thomas. 1991. cDNA encoding the major dust mite allergen Der f II. *Clin. Exp. Allergy* 21:33-38.
- 30 72. Aki T, Kodama T, Fujikawa A, Miura K, Shigeta S, Wada T, Jyo T, Murooka Y, Oka S, Ono K. 1995. Immunochemical characterisation of recombinant and native tropomyosins as a new allergen from the house dust mite *Dermatophagoides farinae*. *J Allergy Clin Immunol* 96:74-83.
73. van Hage-Hamsten, M., T. Bergman, E. Johansson, B. Persson, H. Jornvall, B. Harfast, and S.G.O. Johansson. 1993. N-terminal amino acid sequence of major allergen of the mite *lepidoglyphus destructor* (abst). *J. Allergy Clin. Immunol.* 91:353.
- 35 74. Varela J, Ventas P, Carreira J, Barbas JA, Gimenez-Gallego G, Polo F. Primary structure of Lep d I, the main *Lepidoglyphus destructor* allergen. *Eur J Biochem* 225:93-98. 1994.
75. Schmidt M, van der Ploeg I, Olsson S, van Hage Hamsten M. The complete cDNA encoding the *Lepidoglyphus destructor* major allergen Lep d I. *FEBS Lett* 370:11-14. 1995.
- 40 76. Rautiainen J, Rytönen M, Pelkonen J, Pentikainen J, Perola O, Virtanen T, Zeiler T, Mantylarvi R. BDA20, a major bovine dander allergen characterized at the sequence level is Bos d 2. Submitted.
77. Gjesing B, Lowenstein H. Immunochemistry of food antigens. *Ann Allergy* 53:602. 1984.
78. de Groot, H., K.G.H. Goei, P. van Swieten, and R.C. Aalberse. 1991. Affinity purification of a major and a minor allergen from dog extract: Serologic activity of affinity-purified Can f I and Can f I-depleted extract. *J. Allergy Clin. Immunol.* 87:1056-1065.
- 45 79. Konieczny, A. Personal communication: Immunologic Pharmaceutical Corp.
- 79A. Bulone, V. 1998. Separation of horse dander allergen proteins by two-dimensional electrophoresis. Molecular characterisation and identification of Equ c 2.0101 and Equ c 2.0102 as lipocalin proteins. *Eur J Biochem* 253:202-211.
- 50 79B. Swiss-Prot acc. P81216, P81217.
80. McDonald, B., M. C. Kuo, J. L. Ohman, and L. J. Rosenwasser. 1988. A 29 amino acid peptide derived from rat alpha 2 euglobulin triggers murine allergen specific human T cells (abst). *J. Allergy Clin. Immunol.* 83:251.
- 55 81. Clarke, A. J., P. M. Cissold, R. A. Shawi, P. Beattie, and J. Bishop. 1984. Structure of mouse urinary protein genes: differential splicing configurations in the 3'-non-coding region. *EMBO J* 3:1045-1052.

82. Longbottom, J. L. 1983. Characterization of allergens from the urines of experimental animals. McMillan Press, London, pp. 525-529.
83. Laperche, Y., K. R. Lynch, K. P. Dolans, and P. Feigelsen. 1983. Tissue-specific control of alpha 2u globulin gene expression: constitutive synthesis in submaxillary gland. *Cell* 32:453-460.
- 5 83A. Aukrust L, Borch SM. 1979. Partial purification and characterization of two *Cladosporium herbarum* allergens. *Int Arch Allergy Appl Immunol* 60:68-79.
- 83B. Sward-Nordmo M, Paulsen BS, Wold JK. 1988. The glycoprotein allergen Ag-54 (Cla h II) from *Cladosporium herbarum*. Structural studies of the carbohydrate moiety. *Int Arch Allergy Appl Immunol* 85:288-294.
- 10 84. Shen, et al. *J. Allergy Clin. Immunol.* 103:S157. 1999.
- 84A. Crameri R. Epidemiology and molecular basis of the involvement of *Aspergillus fumigatus* in allergic diseases. *Contrib. Microbiol.* Vol. 2, Karger, Basel (in press).
- 84B. Shen, et al. (manuscript submitted). 1999
- 84C. Shen HD, Ling WL, Tan MF, Wang SR, Chou H, Han SH. Vacuolar serine proteinase: A major allergen of *Aspergillus fumigatus*. 10th International Congress of Immunology, Abstract, 1998.
- 15 85. Kumar, A., L.V. Reddy, A. Sochanik, and V.P. Kurup. 1993. Isolation and characterization of a recombinant heat shock protein of *Aspergillus fumigatus*. *J. Allergy Clin. Immunol.* 91:1024-1030.
86. Teshima, R., H. Ikebuchi, J. Sawada, S. Miyachi, S. Kitani, M. Iwama, M. Irie, M. Ichinoe, and T. Terao. 1993. Isolation and characterization of a major allergenic component (gp55) of *Aspergillus fumigatus*. *J. Allergy Clin. Immunol.* 92:698-706.
- 20 86A. Shen HD, Lin WL, Tsai JJ, Liaw SF, Han SH. 1996. Allergenic components in three different species of *Penicillium*: crossreactivity among major allergens. *Clin Exp Allergy* 26:444-451.
- 86B. Shen, et al. Abstract: The XVIII Congress of the European Academy of Allergology and Clinical Immunology, Brussels, Belgium. 3-7 July 1999.
- 25 87. Shen HD, Liaw SF, Lin WL, Ro LH, Yang HL, Han SH. 1995. Molecular cloning of cDNA coding for the 68 kDa allergen of *Penicillium notatum* using MoAbs. *Clin Exp Allergy* 25:350-356.
88. Shen, H.D., K.B. Choo, H.H. Lee, J.C. Hsieh, and S.H. Han. 1991. The 40 kd allergen of *Candida albicans* is an alcohol dehydrogenase: molecular cloning and immunological analysis using monoclonal antibodies. *Clin. Exp. Allergy* 21:675-681.
- 30 89. Shen, et al. *Clin. Exp. Allergy* (in press), 1999.
90. Woodfolk JA, Wheatley LM, Piyasena RV, Benjamin DC, Platts-Mills TA. 1998. Trichophyton antigens associated with IgE antibodies and delayed type hypersensitivity. Sequence homology to two families of serine proteinases. *J Biol Chem* 273:29489-96.
91. Deuell, B., L.K. Arruda, M.L. Hayden, M.D. Chapman and T.A.E. Platts-Mills. 1991.
- 35 Trichophyton tonsurans Allergen I. *J. Immunol.* 147:96-101.
- 91A. Schmidt M, Zargari A, Holt P, Lindbom L, Hellman U, Whitley P, van der Ploeg I, Harfast B, Scheynius A. 1997. The complete cDNA sequence and expression of the first major allergenic protein of *Malassezia furfur*. *Mol Cell Biol* 17:181-185.
- 91B. Horner WE, Reese G, Lehrer SB. 1995. Identification of the allergen Psi c 2 from the basidiomycete *Psilocybe cubensis* as a fungal cyclophilin. *Int Arch Allergy Immunol* 107:298-300.
- 40 92. Kuchler, K., M. Gmachl, M. J. Sippl, and G. Kreil. 1989. Analysis of the cDNA for phospholipase A2 from honey bee venom glands: The deduced amino acid sequence reveals homology to the corresponding vertebrate enzymes. *Eur. J. Biochem.* 184:249-254.
93. Gmachl, M., and G. Kreil. 1993. Bee venom hyaluronidase is homologous to a membrane protein of mammalian sperm. *Proc. Natl. Acad. Sci. USA* 90:3569-3573.
- 45 94. Habermann, E. 1972. Bee and wasp venoms. *Science* 177:314-322.
95. Jacobson, R.S., and D.R. Hoffman. 1993. Characterization of bumblebee venom allergens (abst). *J. Allergy Clin. Immunol.* 91:187.
96. Arruda LK, Vailes LD, Mann BJ, Shannon J, Fox JW, Vedvick TS, Hayden ML, Chapman MD. Molecular cloning of a major cockroach (*Blattella germanica*) allergen, Bla g 2. Sequence homology to the aspartic proteases. *J Biol Chem* 270:19563-19568. 1995.
- 50 97. Arruda LK, Vailes LD, Hayden ML, Benjamin DC, Chapman MD. Cloning of cockroach allergen, Bla g 4, identifies ligand binding proteins (or calycins) as a cause of IgE antibody responses. *J Biol Chem* 270:31196-31201. 1995.
- 55 98. Arruda LK, Vailes LD, Benjamin DC, Chapman MD. Molecular cloning of German Cockroach (*Blattella germanica*) allergens. *Int Arch Allergy Immunol* 107:295-297. 1995.
- 98A. Wu CH, Lee MF, Liao SC. 1995. Isolation and preliminary characterization of cDNA encoding American cockroach allergens. *J Allergy Clin Immunol* 96: 352-9.

99. Mazur, G., X. Baur, and V. Liebers. 1990. Hypersensitivity to hemoglobins of the Diptera family Chironomidae: Structural and functional studies of their immunogenic/allergenic sites. *Monog. Allergy* 28:121-137.
- 5 100. Soldatova, L., L. Kochoumian, and T.P. King. 1993. Sequence similarity of a hornet (*D. maculata*) venom allergen phospholipase A1 with mammalian lipases. *FEBS Letters* 320:145-149.
101. Lu, G., L. Kochoumian and T.P. King. Whiteface hornet venom allergen hyaluronidase: cloning and its sequence similarity with other proteins (abst.). 1994. *J. Allergy Clin. Immunol.* 93:224.
102. Fang, K. S. F., M. Vitale, P. Fehlner, and T. P. King. 1988. cDNA cloning and primary structure of a white-faced hornet venom allergen, antigen 5. *Proc. Natl. Acad. Sci., USA* 85:895-899.
- 10 103. King, T. P., D. C. Moran, D. F. Wang, L. Kochoumian, and B.T. Chait. 1990. Structural studies of a hornet venom allergen antigen 5. Dol m V and its sequence similarity with other proteins. *Prot. Seq. Data Anal.* 3:263-266.
104. Lu, G., M. Villalba, M.R. Coscia, D.R. Hoffman, and T.P. King. 1993. Sequence analysis and antigen cross reactivity of a venom allergen antigen 5 from hornets, wasps and yellowjackets. *J. Immunol.* 150: 2823-2830.
- 15 105. King, T. P. and Lu, G. 1997. Unpublished data.
- 105A. King TP, Lu G, Gonzalez M, Qian N and Soldatova L. 1996. Yellow jacket venom allergens, hyaluronidase and phospholipase: sequence similarity and antigenic cross-reactivity with their hornet and wasp homologs and possible implications for clinical allergy. *J. Allergy Clin. Immunol.* 98:588-600.
- 20 106. Hoffman, D.R. 1993. Allergens in hymenoptera venom XXV: The amino acid sequences of antigen 5 molecules and the structural basis of antigenic cross-reactivity. *J. Allergy Clin. Immunol.* 92:707-716.
- 25 107. Hoffman, D.R. 1992. Unpublished data.
108. Hoffman, D. R. 1993. The complete amino acid sequence of a yellowjacket venom phospholipase (abst). *J. Allergy Clin. Immunol.* 91:187.
109. Jacobson, R.S., D.R. Hoffman, and D.M. Kemeny. 1992. The cross-reactivity between bee and vespid hyaluronidases has a structural basis (abst). *J. Allergy Clin. Immunol.* 89:292.
- 30 110. Hoffman, D.R. 1993. Allergens in Hymenoptera venom XXIV: The amino acid sequences of imported fire ant venom allergens Sol i II, Sol i III, and Sol i IV. *J. Allergy Clin. Immunol.* 91:71-78.
111. Schmidt, M., R.B. Walker, D.R. Hoffman, and T.J. McConnell. 1993. Nucleotide sequence of cDNA encoding the fire ant venom protein Sol i II. *FEBS Letters* 319:138-140.
112. Elsayed S, Bennich H. The primary structure of Allergen M from cod. *Scand J Immunol* 3:683-686, 1974.
- 35 113. Elsayed S, Aas K, Sletten K, Johansson SGO. Tryptic cleavage of a homogeneous cod fish allergen and isolation of two active polypeptide fragments. *Immunochemistry* 9:647-661, 1972.
114. Hoffman, D. R. 1983. Immunochemical identification of the allergens in egg white. *J. Allergy Clin. Immunol.* 71:481-486.
- 40 115. Langeland, T. 1983. A clinical and immunological study of allergy to hen's egg white. IV. specific IgE antibodies to individual allergens in hen's egg white related to clinical and immunological parameters in egg-allergic patients. *Allergy* 38:493-500.
116. Daul, C.B., M. Slattery, J.E. Morgan, and S.B. Lehrer. 1993. Common crustacea allergens: identification of B cell epitopes with the shrimp specific monoclonal antibodies. In: "Molecular Biology and Immunology of Allergens" (D. Kraft and A. Schon, eds.). CRC Press, Boca Raton. pp. 291-293.
- 45 117. K.N. Shanti, B.M. Martin, S. Nagpal, D.D. Metcalfe, P.V. Subba Rao. 1993. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J. Immunol.* 151:5354-5363.
- 50 117A. M. Miyazawa, H. Fukamachi, Y. Inagaki, G. Reese, C.B. Daul, S.B. Lehrer, S. Inouye, M. Sakaguchi. 1996. Identification of the first major allergen of a squid (*Todarodes pacificus*). *J. Allergy Clin. Immunol.* 98:948-953.
- 117B A. Lopata et al. 1997. Characteristics of hypersensitivity reactions and identification of a unique 49 kDa IgE binding protein (Hal-m-1) in Abalone (*Haliotis midae*). *J. Allergy Clin. Immunol.* Submitted
- 55 118. Monsalve, R.I., M.A. Gonzalez de la Pena, L. Menendez-Arias, C. Lopez-Otin, M. Villalba, and R. Rodriguez. 1993. Characterization of a new mustard allergen. *Bra j IE. Detection of an allergenic epitope. Biochem. J.* 293:625-632.

119. Mena, M., R. Sanchez-Monge, L. Gomez, G. Salcedo, and P. Carbonero. 1992. A major barley allergen associated with baker's asthma disease is a glycosylated monomeric inhibitor of insect alpha-amylase: cDNA cloning and chromosomal location of the gene. *Plant Molec. Biol.* 20:451-458.
120. Menendez-Arias, L., I. Moneo, J. Dominguez, and R. Rodriguez. 1988. Primary structure of the major allergen of yellow mustard (*Sinapis alba* L.) seed, Sin a I. *Eur. J. Biochem.* 177:159-166.
121. Gonzalez R, Varela J, Carreira J, Polo F. Soybean hydrophobic protein and soybean hull allergy. *Lancet* 346:48-49, 1995.
122. Christie, J. F., B. Dunbar, I. Davidson, and M. W. Kennedy. 1990. N-terminal amino acid sequence identity between a major allergen of *Ascaris lumbricoides* and *Ascaris suum* and MHC-restricted IgE responses to it. *Immunology* 69:596-602.
123. Czuppon AB, Chen Z, Rennert S, Engelke T, Meyer HE, Heber M, Baur X. The rubber elongation factor of rubber trees (*Hevea brasiliensis*) is the major allergen in latex. *J Allergy Clin Immunol* 92:690-697, 1993.
124. Attanayaka DPSTG, Kekwick RGO, Franklin FCH. 1991. Molecular cloning and nucleotide sequencing of the rubber elongation factor gene from *hevea brasiliensis*. *Plant Mol Biol* 16:1079-1081.
125. Chye ML, Cheung KY. 1995. (1,3-glucanase is highly expressed in Laticifers of *Hevea brasiliensis*. *Plant Mol Biol* 26:397-402.
126. Alenius H, Palosuo T, Kelly K, Kurup V, Reunala T, Makinen-Kiljunen S, Turjanmaa K, Fink J. 1993. IgE reactivity to 14-kD and 27-kD natural rubber proteins in Latex-allergic children with Spina bifida and other congenital anomalies. *Int Arch Allergy Immunol* 102:61-66.
127. Yeang HY, Cheong KF, Sunderasan E, Hamzah S, Chew NP, Hamid S, Hamilton RG, Cardoso MJ. 1996. The 14.6 kD (REF, Hev b 4) and 17.1 kD (Hev b 5) rubber particle proteins are recognized by IgE from Spina Bifida patients with Latex allergy. *J Allerg Clin Immunol* in press.
128. Sunderasan E, Hamzah S, Hamid S, Ward MA, Yeang HY, Cardoso MJ. 1995. Latex B-serum (-1,3-glucanase (Hev b 2) and a component of the microhelix (Hev b 4) are major Latex allergens. *J nat Rubb Res* 10:82-99.

We claim:

THIS PAGE BLANK (USPTO)

1. A composition comprising:
 - i) an oligonucleotide comprising a nucleotide sequence 5' purine-purine-C-G-pyrimidine-pyrimidine 3' wherein C and G are unmethylated; and
 - ii) at least one antigen derived from an allergen that causes anaphylaxis.
2. The composition of claim 1, wherein the antigen is derived from the group consisting of: food allergens, venom allergens, latex allergens, and drug allergens.
3. The composition of claim 1, wherein the antigen is a peptide, polypeptide, or protein.
4. The composition of claim 1, wherein the at least one antigen comprises a collection of peptides.
5. The composition of claim 4, wherein the collection of peptides contains peptides having overlapping amino acid sequences.
6. The composition of claim 4, wherein the collection of peptides contains peptides without overlapping amino acid sequences.
7. The composition of claim 4, wherein the collection comprises peptides wherein each peptide has fewer than two IgE binding sites.
8. The composition of claim 1, wherein the antigen comprises a polypeptide having an amino acid sequence found in a food allergen.
9. The composition of claim 8, wherein the polypeptide is modified to reduce IgE binding sites.
10. The composition of claim 8 or 9, wherein the polypeptide is derived from a protein from a peanut, milk or eggs.

11. The composition of claim 1, wherein the oligonucleotide comprises a nucleotide sequence selected from the group consisting of: 5' TCAACGTT 3', 5' GACGTT 3', 5' AGCGTT 3', 5' AACGCT 3', 5' AACGAT 3', 5' TCAACGTT 3', 5' GACGTT 3', 5' AGACGT 3', 5' AACGCT 3', 5' TGACGTT 3', 5' TAGACGT 3', 5' TAACGCT 3', 5' TCGTCGTTTT 3', and 5' TCGTCGTTTTGTCGTTTTGTCGTT 3', wherein CpG dinucleotides are unmethylated.
12. A method of treating an individual with an allergy comprising steps of:
- i) administering to an individual a composition comprising:
- a) an oligonucleotide comprising a sequence 5' purine-purine-C-G-pyrimidine-pyrimidine 3' wherein C and G are unmethylated; and
- b) at least one antigen derived from an allergen that causes anaphylaxis.
13. The method of claim 12, wherein the antigen is derived from the group consisting of: food allergens, venom allergens, latex allergens, and drug allergens.
14. The method of claim 12, wherein the antigen is a peptide, polypeptide or protein.
15. The method of claim 12, wherein the at least one antigen comprises a collection of peptides.
16. The method of claim 15, wherein the collection of peptides contains peptides with overlapping amino acid sequences.
17. The method of claim 15, wherein the collection of peptides contains peptides without overlapping amino acid sequences.
18. The method of claim 16 or 17, wherein the collection comprises peptides wherein each peptide has fewer than two IgE binding sites.

19. The method of claim 12, wherein the at least one antigen comprises a polypeptide having an amino acid sequence derived from a food allergen.
20. The method of claim 19, wherein the polypeptide is modified to reduce IgE binding sites.
21. The method of claim 12, wherein the at least one antigen is derived from a protein from a peanut, milk or eggs.
22. The method of claim 12, wherein the oligonucleotide comprises a nucleotide sequence selected from the group consisting of: 5' TCAACGTT 3', 5' GACGTT 3', 5' AGCGTT 3', 5' AACGCT 3', 5' AACGAT 3', 5' TCAACGTT 3', 5' GACGTT 3', 5' AGACGT 3', 5' AACGCT 3', 5' TGACGTT 3', 5' TAGACGT 3', 5' TAACGCT 3', 5' TCGTCGTTTT 3', and 5' TCGTCGTTTTGTCGTTTTGTCGTT 3', wherein CpG dinucleotides are unmethylated.
23. A method of treating an individual with an allergy comprising steps of:
- i) administering to an individual a composition comprising:
 - a) an oligonucleotide comprising a sequence 5' purine-purine-C-G-pyrimidine-pyrimidine 3' wherein C and G are unmethylated; and
 - b) a peptide, polypeptide or protein that is non-allergenic;
 - and
 - ii) administering to an individual a composition comprising at least one antigen.
24. The method of claim 23, wherein the antigen is an antigen that causes anaphylaxis.
25. The method of claim 23, wherein the antigen is derived from the group consisting of: food allergens, venom allergens, latex allergens, and drug allergens.

26. The method of claim 23, wherein the antigen is a peptide, polypeptide or protein.
27. The method of claim 23, wherein the at least one antigen comprises a collection of peptides.
28. The method of claim 27, wherein the collection of peptides contain peptides with overlapping amino acid sequences.
29. The method of claim 27, wherein the collection of peptides contain peptides without overlapping amino acid sequences.
30. The method of claim 28 or 29, wherein the collection comprises peptides wherein each peptide has fewer than two IgE binding sites.
31. The method of claim 23, wherein the at least one antigen comprises a polypeptide having an amino acid sequence derived from a food allergen.
32. The method of claim 31, wherein the polypeptide is modified to reduce IgE binding sites.
33. The method of claim 23, wherein the at least one antigen is derived from a protein from a peanut.
34. The method of claim 23, wherein the oligonucleotide comprises a nucleotide sequence selected from the group consisting of: 5' TCAACGTT 3', 5' GACGTT 3', 5' AGCGTT 3', 5' AACGCT 3', 5' AACGAT 3', 5' TCAACGTT 3', 5' GACGTT 3', 5' AGACGT 3', 5' AACGCT 3', 5' TGACGTT 3', 5' TAGACGT 3', 5' TAACGCT 3', 5' TCGTCGTTTT 3', and 5' TCGTCGTTTTGTCGTTTTGTCGTT 3', wherein CpG dinucleotides are unmethylated.
35. The method of claim 23, further comprising:

iii) administering to a second individual,

a) an oligonucleotide comprising a sequence 5' purine-purine-C-G-pyrimidine-pyrimidine 3' wherein C and G are unmethylated; and

b) a peptide, polypeptide or protein that is non-allergenic;

5 and

iv) administering to the second individual a composition comprising at least one antigen.

36. The method of claim 23, further comprising administering to an individual a
10 composition comprising at least one antigen, wherein the antigen is different from the antigen in step ii).

37. A pharmaceutical composition comprising:

i) an oligonucleotide comprising a sequence 5' purine-purine-C-G-pyrimidine-
15 pyrimidine 3' wherein C and G are unmethylated; and

ii) at least one antigen derived from an allergen that causes anaphylaxis.

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)